AN IMPROVED METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE ON A CELLULAR RESPONSE.

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SUMMARY OF THE INVENTION

The present invention relates to an improved method and tools for extracting quantitative information relating to an influence on a cellular response, in particular an influence caused by contacting or incubating the cell with a substance influencing a cellular response, wherein the cellular response is manifested in redistribution of at least one component in the cell. In particular, the invention relates to an improved method for extracting the quantitative information relating to an influence on an intracellular pathway involving redistribution of at least one component associated with the pathway. The method of the invention may be used as a very efficient procedure for testing or discovering the influence of a substance on a physiological process, for example in connection with screening for new drugs, testing of substances for toxicity, identifying drug targets for known or novel drugs. In particular, the present invention relates to an improved method for parallelisation of the testing procedure so that a large number of substances can be tested simultaneously using commercially available instrumentation. The invention also describes several ways of contacting the cells with a substance influencing a cellular response and modifications made to the actual cells before, during or after contacting the cells with these substances as to improve the applicability and use of the method for extracting quantitative information relating to influence on an intracellular pathway in a highly parallel fashion. Other valuable uses of the method and technology of the invention will be apparent to the skilled person on the basis of the following disclosure. In a particular embodiment of the invention, the present invention relates to a method of detecting intracellular translocation or redistribution of biologically active polypeptides, preferably an enzyme, affecting intracellular processes, and a DNA construct and a cell for use in the method.

BACKGROUND OF THE INVENTION

Intracellular pathways are tightly regulated by a cascade of components that undergo modulation in a temporally and spatially characteristic manner. Several disease states can be attributed to altered activity of individual signalling components (i.e. protein

kinases, protein phosphatases, transcription factors). These components therefore render themselves as attractive targets for therapeutic intervention.

Protein kinases and phosphatases are well-described components of several intracellular signalling pathways. The catalytic activity of protein kinases and phosphatases are assumed to play a role in virtually all regulatable cellular processes. Although the involvement of protein kinases in cellular signalling and regulation have been subjected to extensive studies, detailed knowledge on e.g. the exact timing and spatial characteristics of signalling events is often difficult to obtain due to lack of a convenient technology.

The measurement of the activity of intracellular enzymes, such as kinases and phosphatases, can be performed by well-established procedures, both manually and in various automated forms, at throughput rates which make these measurements useful in the search for new drug candidates. In addition to measures of activity, measures of the distribution of these and other enzymes in the cell has proven useful, and established techniques exist for this type of measurement as well. Protein kinases often show a specific intracellular distribution before, during and after activation. Monitoring the translocation processes and/or redistribution of individual protein kinases or subunits thereof is thus likely to be indicative of their functional activity. A connection between translocation and catalytic activation has been shown for protein kinases like the diacyl glycerol (DAG)-dependent protein kinase C (PKC), the cAMP-dependent protein kinase (PKA) [(DeBernardi *et al.*1996)] and the mitogen-activated-protein kinase Erk-1 [(Sano *et al.*1995)]. Such methods of detection of intracellular localisation/activity of protein kinases and phosphatases include immunoprecipitation, Western blotting and immunocytochemical detection.

One aspect of the function of intracellular enzymes which has not been characterised so thoroughly is the redistribution of those enzymes. The importance of subcellular redistribution of enzymes as a mechanism of enzyme specificity, and of the general importance of the measurement of subcellular redistribution as a tool for identifying novel drug targets and searching for drug candidates which influence those targets, is disclosed in: A METHOD FOR EXTRACTING QUANTITATIVE INFORMATION RELATING TO AN INFLUENCE ON A CELLULAR RESPONSE ,the contents of which

were part of the priority application, and which, as WO9845704 has been published during the priority year, are hereby incorporated herein by reference.

While the redistribution of subcellular components is known to be important, the measurement of this phenomenon in real time has not been widely exploited. This is primarily due to the lack of a suitable technique. There is essentially only one direct technique: the microscopic imaging of cells in which the subcellular component of interest has been labelled in such a way that it can be visualised and recorded by the microscopic imaging system, using for example a video or scientific CCD camera and appropriate software for collecting and storing the images. Novel ways of monitoring specific modulation of intracellular pathways in intact, living cells is assumed to provide new opportunities in drug discovery, functional genomics, toxicology, patient monitoring etc.

Recently it was discovered that Green Fluorescent Protein (GFP) expressed in many different cell types, including mammalian cells, became highly fluorescent [(Chalfie *et al.* 1994)]. WO95/07463 describes a cell capable of expressing GFP and a method for detecting a protein of interest in a cell based on introducing into a cell a DNA molecule having DNA sequence encoding the protein of interest linked to DNA sequence encoding a GFP such that the protein produced by the DNA molecule will have the protein of interest fused to the GFP, then culturing the cells in conditions permitting expression of the fused protein and detecting the location of the fluorescence in the cell, thereby localizing the protein of interest in the cell. However, examples of such fused proteins are not provided, and the use of fusion proteins with GFP for detection or quantitation of translocation or redistribution of biologically active polypeptides affecting intracellular processes upon activation, such as proteins involved in signalling pathways, e.g. protein kinases or phosphatases, has not been suggested. WO 95/07463 further describes cells useful for the detection of molecules, such as hormones or heavy metals, in a biological sample, by operatively linking a regulatory element of the gene which is affected by the molecule of interest to a GFP, the presence of the molecules will affect the regulatory element which in turn will affect the expression of the GFP. In this way the gene encoding GFP is used as a reporter gene in a cell which is constructed for monitoring the presence of a specific molecular identity.

Green Fluorescent Protein has been used in an assay for the detection of translocation of the glucocorticoid receptor (GR) [(Carey, KL *et al.* 1996)]. A GR-S65TGFP fusion has been used to study the mechanisms involved in translocation of the glucocorticoid receptor (GR) in response to the agonist dexamethasone from the cytosol, where it is present in the absence of a ligand, through the nuclear pore to the nucleus where it remains after ligand binding. The use of a GR-GFP fusion enables real-time imaging and quantitation of nuclear/cytoplasmic ratios of the fluorescence signal. A similar genetic construct has been used to follow and quantify dexamethasone induced translocation of GR to the nucleus in HeLa cells [(Guiliano, K.A *et al.* 1997)] in a system called Array Scan™ (WO 97/45730) designed for automated drug screening. Recently, several other investigators have demonstrated that tagging a specific protein (or part of a protein) involved in an intracellular signalling pathway with GFP provides a new means to measure and quantify the influence of substances on this pathway. The concept has been shown to work both for cytoplasmic to nuclear translocation of the androgen receptor [(Georget V *et al.* 1997)] and transcription factors such as NF-ATc [(Beals CR *et al. 1997*)] in analogy with what has already been described for GR above. Another relevant example is a β-arrestin – GFP construct that was shown to report on activation of G-protein coupled receptors by translocating from the cytosol to the plasma membrane [(Barak LS *et al.* 1997)]. Finally, it has also been demonstrated that attaching GFP to a smaller part of a protein like the pleckstrin homology domain of phospholipase C δ 1 [(Stauffer TP *et al.* 1998)] and a cysteine-rich domain of PKC γ [(Oancea E *et al.* 1998)] can be used to report on an influence from a substance by quantifying their redistribution within the cells during activation of the specific signalling pathway to which they belong.

Many currently used screening programmes designed to find compounds that affect protein kinase activity are based on measurements of kinase phosphorylation of artificial or natural substrates, receptor binding and/or reporter gene expression. The interest in fluorescence measurements as the basis for future high-throughput drug screening has however increased dramatically over the last few years [(Silverman L *et al.* 1998)]. Of particular interest to the present invention is a scanning laser imager for rapid screening of fluorescence changes in living cells [(Schroeder K & Neagle B 1996)] currently offered commercially by Molecular Devices, Inc. as the FLIPR™.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an important new dimension in the investigation of cellular systems involving redistribution in that the invention provides quantification of the redistribution responses or events caused by an influence, typically contact with a chemical substance or mixture of chemical substances, but also changes in the physical environment, in a massively parallel fashion. The quantification makes it possible to set up meaningful relationships, expressed numerically, or as curves or graphs, between the influences (or the degree of influences) on cellular systems and the redistribution response. This is highly advantageous because, as has been found, the quantification can be achieved in both a fast and reproducible manner, and - what is perhaps even more important - the systems which become quantifiable utilising the method of the invention are systems from which enormous amounts of new information and insight can be derived.

The present screening assays have the distinct advantage over other screening assays, e.g., receptor binding assays, enzymatic assays, and reporter gene assays, in providing a system in which biologically active substances with completely novel modes of action, e.g. inhibition or promotion of redistribution/translocation of a biologically active polypeptide as a way of regulating its action rather than inhibition/activation of enzymatic activity, can be identified in a way that insures very high selectivity to the particular isoform of the biologically active polypeptide and further development of compound selectivity versus other isoforms of the same biologically active polypeptide or other components of the same signalling pathway.

In one of its broadest aspects, the invention relates to an improved method, with higher throughput compared to previous methods, for extracting quantitative information relating to an influence on a cellular response, the method comprising recording variation, caused by the influence on mechanically intact living cells, in spatially distributed light emitted from a luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence, the association resulting in a modulation of the luminescence characteristics of the luminophore, detecting and recording the variation in spatially distributed light from the luminophore as

a change in fluorescence intensity using an instrument designed to measure changes in fluorescence intensity, and processing the recorded variation in the spatially distributed light to provide quantitative information correlating the spatial distribution or change in the spatial distribution to the degree of the influence. In one aspect of the present invention the mechanically intact living cell is permeabilised at some time after the influence has begun but during or before the actual experimental recording. In another aspect, the present invention relates to an improved method for extracting quantitative information relating to an influence on a cellular response, the method comprising recording variation, caused by the influence on permeabilised living cells, in spatially distributed light emitted from a luminophore, the luminophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or of being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence, the association resulting in a modulation of the luminescence characteristics of the luminophore, detecting and recording the spatially distributed light from the luminophore as a change in fluorescence intensity using an instrument designed to measure changes in fluorescence intensity, and processing the recorded variation in the spatially distributed light to provide quantitative information correlating the spatial distribution or change in the spatial distribution to the degree of the influence. In a preferred embodiment of the invention the luminophore, which is present in the cells, is capable of being redistributed by modulation of an intracellular pathway, in a manner which is related to the redistribution of at least one component of the intracellular pathway. In another preferred embodiment of the invention, the luminophore is a fluorophore.

Typically the cell and/or cells are mechanically intact and alive throughout the experiment. In another embodiment of the invention, the cells are fixed at a point in time after the application of the influence at which the response has been predetermined to be significant, and the recording is made at an arbitrary later time. In another embodiment the cell and/or cells are mechanically intact and alive throughout the experiment but are mechanically or chemically disrupted or permeabilised as the initial step of experimental analysis. In another aspect of the invention the cells have their plasma membrane permanently and stably permeabilised before the initiation of the experiment in such a way that the plasma membrane stays permeable during the experiment. This allows the components of intracellular pathways to be contacted by

substances that are not normally permeating the cell plasma membrane such as peptides, proteins and hydrophilic organic compounds..

The mechanically intact or permeabilised living cells could be selected from the group consisting of fungal cells, such as yeast cells; invertebrate cells including insect cells; and vertebrate cells, such as mammalian cells. These cells are incubated at a temperature of 30°C or above, preferably at a temperature of from 32°C to 39°C, more preferably at a temperature of from 35°C to 38°C, and most preferably at a temperature of about 37°C during the time period over which the influence is observed. In one aspect of the invention the mechanically intact or permeabilised living cell is part of a matrix of identical or non-identical cells. In one embodiment of the invention the cells comprise a group or groups of cells contained within a spatial limitation or spatial limitations. In one embodiment, the cells comprise multiple groups of cells that are qualitatively the same but subjected to different influences. In another embodiment, the cells comprise multiple groups of cells that are qualitatively different but subjected to the same influence.

In one embodiment of the invention the spatial limitations are domains defined on a substrate on which the cells are present. The spatial limitations may be arranged in one or more arrays on a common carrier. The spatial limitations may be wells in a plate of microtiter type, such that 96, 384, 864 and 1536 wells are situated on the common carrier. In another embodiment the spatial limitations are wells in a plate of a format different from the microtiter type. In one embodiment of the invention the domains are established by the presence of the cells on the substrate in a pattern that defines the domains. In another aspect of the invention, the domains are instead established by the spatial pattern or array of the influence or influences as it/they are applied to or contacted by the cells. This aspect is thoroughly disclosed in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which. as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference. Briefly, in this aspect of the invention the mechanically intact or permeabilised living cells are part of a continuous or discontinuous sheet of cells cultured on an optically clear flat surface typically optimised for cell culture. The optically clear and flat surface may be a porous membrane that may allow cellular processes to grow through the membrane pores and may allow directed capillary flow of fluid through the pores.

A cell used in the present invention should contain a nucleic acid construct encoding a fusion polypeptide as defined herein and be capable of expressing the sequence encoded by the construct. The cell is a eukaryotic cell selected from the group consisting of fungal cells, such as yeast cells; invertebrate cells including insect cells; vertebrate cells such as mammalian cells. The preferred cells are mammalian cells.

In another aspect of the invention the cells could be from an organism carrying in at least one of its component cells a nucleic acid sequence encoding a fusion polypeptide as defined herein and be capable of expressing said nucleic acid sequence. The organism is selected from the group consisting of unicellular and multicellular organisms, such as a mammal.

The luminophore is the component that allows the redistribution to be visualised and/or recorded by emitting light in a spatial distribution related to the degree of influence. The term redistribution is intended to cover all aspects of a change in spatial location, such as a translocation of the luminophore or other components. In one embodiment of the invention, the luminophore is capable of being redistributed in a manner that is physiologically relevant to the degree of the influence. In another embodiment, the luminophore is capable of associating with a component that is capable of being redistributed in a manner that is physiologically relevant to the degree of the influence. In another embodiment, a correlation between the redistribution of the luminophore and the degree of the influence could be determined experimentally. In a preferred aspect of the invention, the luminophore is capable of being redistributed in substantially the same manner as the at least one component of an intracellular pathway. In another embodiment of the invention, the luminophore is capable of being quenched upon spatial association with a component that is redistributed by modulation of the pathway, the quenching being measured as a change in the intensity of the luminescence. In another embodiment of the invention, the luminophore is stationary but may have a certain spatial distribution, and interacts with at least one component that is capable of being redistributed in a manner which is physiologically relevant to the degree of the influence, in such a way that one or more luminescence characteristics of the luminophore is/are modulated as the component moves closer to, or farther from, the luminophore.

The luminophore could be a fluorophore. In a preferred embodiment of the invention, the luminophore is a polypeptide encoded by and expressed from a nucleotide sequence

harboured in the cells. The luminophore could be a hybrid polypeptide comprising a fusion of at least a portion of each of two polypeptides one of which comprises a luminescent polypeptide and the other one of which comprises a biologically active polypeptide, as defined herein.

The luminescent polypeptide could be a GFP as defined herein or could be selected from the group consisting of green fluorescent proteins having the F64L mutation as defined herein such as F64L-GFP, F64L-Y66H-GFP, F64L-S65T-GFP, and EGFP. The GFP could be N- or C-terminally tagged, optionally via a peptide linker, to the biologically active polypeptide or a part or a subunit thereof. The fluorescent probe could be a component of an intracellular signalling pathway. The probe is coded for by a nucleic acid construct.

In one aspect of the invention the pathway of investigation is an intracellular signalling pathway.

In a preferred embodiment of the invention, the influence could be contact between the group or groups of mechanically intact or permeabilised living cells and a chemical substance, and/or incubation of the group or groups of mechanically intact or permeabilised living cells with a chemical substance in solution. In one aspect of the invention that is thoroughly described in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference, the chemical substances are attached to an underlying matrix. In this aspect, the chemical substances may also be produced and secreted from, or attached to the plasma membrane surfaces of, a sheet of genetically engineered cells. In this aspect of the invention the chemical substances may also have been separated two-dimensionally in a non-denaturing gel using electrophoresis and the gel is directly put in close proximity or direct contact with the mechanically intact or permeabilised living cells so that the chemical substances can contact the cells through diffusion or convection.

The influence will modulate the intracellular processes. In one aspect the modulation could be an activation of the intracellular processes. In another aspect the modulation could be a deactivation of the intracellular processes. In yet another aspect, the

influence could inhibit or promote the redistribution without directly affecting the metabolic activity of the component of the intracellular processes.

In one embodiment the invention is used to establish a dose-response relationship for one or many chemical substances. In one embodiment the invention is used as a basis for a screening program, where the effect of unknown influences such as a compound library, can be compared to influence of known reference compounds under standardised conditions.

In addition to the intensity, there are several parameters of fluorescence or luminescence that can be modulated by the effect of the influence on the underlying cellular phenomena, and can therefore be used in the invention. Some examples are resonance energy transfer, fluorescence lifetime, polarisation, and wavelength shift. Each of these methods requires a particular kind of filter in the emission light path to select the component of the light desired and reject other components. The recording of property of light could be in the form of an ordered array of values such as a CCD array or a vacuum tube device such as a vidicon. In addition, the translational mobility, or freedom of movement, of the luminophore attached to the protein of interest can be an important property affected by the influence on the underlying cellular phenomena, and can therefore be used in the invention.

In one embodiment of the invention, the spatially distributed light emitted by a luminophore is detected by a change in the resonance energy transfer between the luminophore and another luminescent entity capable of delivering energy to the luminophore, each of which has been selected or engineered to become part of, bound to or associated with particular components of the intracellular pathway. In this embodiment, either the luminophore or the luminescent entity capable of delivering energy to the luminophore undergoes redistribution in response to an influence. The resonance energy transfer would be measured as a change in the intensity of emission from the luminophore, preferably sensed by a single channel photodetector that responds only to the average intensity of the luminophore in a non-spatially resolved fashion.

In one embodiment of the invention, the spatially distributed light emitted by a luminophore includes the case of uniform spatial distribution of the light.

In one aspect of the invention, the luminophore is a fluorophore which redistributes through a non-homogenous excitation light field, resulting in a change in the intensity of the light emitted from the luminophore as a result of the change in the amount of excitation light intensity at different points in the field.

In one embodiment of the invention, the recording of the spatially distributed light could be made at a single point in time after the application of the influence. In another embodiment, the recording could be made at two points in time, one point being before, and the other point being after the application of the influence. The result or variation is determined from the change in fluorescence compared to the fluorescence measured prior to the influence or modulation. In another embodiment of the invention, the recording could be performed at a series of points in time, in which the application of the influence occurs at some time after the first time point in the series of recordings, the recording being performed, e.g., with a predetermined time spacing of from 0.1 seconds to 1 hour, preferably from 1 to 60 seconds, more preferably from 1 to 30 seconds, in particular from 1 to 10 seconds, over a time span of from 1 second to 12 hours, such as from 10 seconds to 12 hours, e.g., from 10 seconds to one hour, such as from 60 seconds to 30 minutes or 20 minutes. The result or variation is determined from the change in fluorescence over time. The result or variation could also be determined as a change in the spatial distribution of the fluorescence over time.

In one embodiment the recording comprises a time series of total luminescence of the cells of one or several of the spatial limitations. In one embodiment the signal from all of the spatial limitations, one at a time, is measured by a recording being made in the individual spatial limitations by means of an apparatus to sequentially position each one of the limitations in the field of view of the detector and repeating the positioning and measurement process until all of the spatial limitations have been measured. The detector may be a photomultiplier tube. In a preferred embodiment of the present invention more than one spatial limitation is measured simultaneously. This may be done by means of a one- or two-dimensional array detector, whereby the multiple spatial limitations are imaged onto the array detector such that discrete subsets of the detecting units (pixels) in the array detector measure the signal from one and only one of the multiple spatial limitations, the signal from any one spatial limitation being the combined signal from those pixels that receive the image from one of the spatial limitations. This

array detector may be a linear diode array, a video camera (according to any present or future standards and definitions of image acquisition and transmission) or a charge transfer device such as a charge-coupled device (CCD). In one embodiment the recording of signal requires illumination of the multiple spatial limitations to excite the luminophores so that they emit light. In one embodiment all of the spatial limitations are simultaneously illuminated during the measurement. In another embodiment the spatial limitations are singly illuminated only during the time in which they are being measured. In a preferred embodiment the illumination is provided by a laser that is scanned in a raster fashion over some or all of the spatial limitations being measured. The scanning may take place at a rate that is substantially faster than the measurement process such that the illumination appears to the measurement process to be continuous in time and spatially uniform over the region being measured.

The recording of spatially distributed luminescence emitted from the luminophore is performed by an apparatus for measuring the distribution of fluorescence in the cells, and thereby any change in the distribution of fluorescence in the cells, which includes at a minimum the following component parts: (a) a light source, (b) a method for selecting the wavelength(s) of light from the source which will excite the luminescence of the luminophore, (c) a device which can rapidly block or pass the excitation light into the rest of the system, (d) a series of optical elements for conveying the excitation light to the specimen, collecting the emitted fluorescence in a spatially resolved fashion, and forming an image from this fluorescence emission (or another type of intensity map relevant to the method of detection and measurement), (e) a bench or stand which holds the container of the cells being measured in a predetermined geometry with respect to the series of optical elements, (f) a detector to record the spatially resolved fluorescence in the form of an image, (g) a computer or electronic system and associated software to acquire and store the recorded images, and to compute the degree of redistribution from the recorded images.

In a preferred embodiment of the invention the apparatus system is automated. In one embodiment the components in d and e mentioned above comprise a fluorescence microscope. In one embodiment the component in f mentioned above is a CCD camera. In one embodiment the component in f mentioned above is an array of photomultiplier tubes/devices.

In one embodiment the image is formed and recorded by an optical scanning system.

In one embodiment the optical scanning system is used to illuminate the bottom of a plate of microtiter type so that a time-resolved recording of changes in luminescence or fluorescence can be made from all spatial limitations simultaneously.

In a preferred embodiment the actual luminescence or fluorescence measurements are made in a FLIPR™ instrument, commercially available from Molecular Devices, Inc.

In one embodiment of the invention the actual fluorescence measurements are made in a standard type of fluorometer for plates of microtiter type (fluorescence plate reader).

In one embodiment a liquid addition system is used to add a known or unknown compound to any or all of the cells in the cell holder at a time determined in advance. Preferably, the liquid addition system is under the control of the computer or electronic system. Such an automated system can be used for a screening program due to its ability to generate results from a larger number of test compounds than a human operator could generate using the apparatus in a manual fashion.

The methods whereby the detector layer of cells are physically contacted by the compounds can also be of another conceptual type where the compounds are delivered to the cells through a porous membrane by convection/diffusion or by directly contacting compounds attached to an inorganic or organic support (such as glass, plastic or the plasma membrane of intact living cells) with the cells. These methods are thoroughly described in "Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference, but are also outlined in the following paragraphs.

In one aspect of the present invention where the detector layer of cells is a continuous or discontinuous sheet of cells without any separation into test units or wells. The compounds are printed onto a nonabsorbent sheet of porous material as a solution in solvent and allowed to dry. This printed sheet of compounds then defines the test pattern for the experiment as it is brought down in close proximity to or in direct contact with the underlying detector layer of cells. The compounds, now dissolved by the fluid layer on

the cells, is brought in contact with the cells through the pores of the membrane by convection. The porous membrane onto which the compounds are printed is optically clear and preferably composed as stated in"Method and apparatus for high density format screening for bioactive molecules" the contents of which were part of the priority application, and which, as WO99/35496 has been published during the priority year, are hereby incorporated herein by reference. In another embodiment of this aspect of the present invention the detector layer of cells is a continuous or discontinuous sheet of cells, without any separation into test units or wells, growing on a porous and optically clear membrane preferably of the types mentioned above. The porous membrane may allow the cells to send cellular processes through the pores of the membrane. The compounds are printed onto an optically clear substratum such as glass, plastic or quartz as solutions in solvent and allowed to dry. At the time of the experiment the cell sheet on the membrane, surrounded by a thin film of fluid, is layered ontop of the printed compound pattern. The compounds then dissolve and contact the cells via diffusion and convection. The compounds may be made using combinatorial chemistry techniques, and may be peptides. The compounds may be covalently attached to the optically clear substratum or porous membrane. The compounds may also be proteins, polypeptides or peptides secreted by or attached to the plasma membrane of genetically modified cells growing as a continuous or discontinuous sheet on a flat optically clear surface or an optically clear porous membrane.

The recording of the variation or result with respect to light emitted from the luminophore is performed by recording the spatially distributed light as one or more digital images, and the processing of the recorded variation to reduce it to one or more numbers representative of the degree of redistribution comprises a digital image processing procedure or combination of digital image processing procedures. The quantitative information which is indicative of the degree of the cellular response to the influence or the result of the influence on the intracellular pathway is extracted from the recording or recordings according to a predetermined calibration based on responses or results, recorded in the same manner, to known degrees of a relevant specific influence. This calibration procedure is developed according to principles described below (Developing an Image-based Assay Technique). Specific descriptions of the procedures for particular assays are given in the examples.

While the stepwise procedure necessary to reduce the image or images to the value representative of the response caused by the influence is particular to each assay, the individual steps are generally well-known methods of image processing. Some examples of the individual steps are point operations such as subtraction, ratioing, and thresholding, digital filtering methods such as smoothing, sharpening, and edge detection, spatial frequency methods such as Fourier filtering, image cross-correlation and image autocorrelation, object finding and classification (blob analysis), and colour space manipulations for visualisation. In addition to the algorithmic procedures, heuristic methods such as neural networks may also be used. In a preferred embodiment of the invention, a dose-response relationship is established based on quantification of the responses caused by a particular influence, representative of the underlying intracellular signalling process, using the methods described above and in the examples. The dose-response relationship for the particular influence is then compared to the dose-response relationship obtained by performing the same assay in an instrument which allows parallel monitoring of all wells in a microtiter plate such as a FLIPR™ or an ordinary fluorescence plate reader for microtiter plates. If a good correlation between the dose-response relationships obtained from the two different measurement systems is obtained, it can be said that the parallel measurement mode has been validated (see examples 8, 9, 10 and 11). This implies that it can be used as the primary basis for a screening assay with the potential benefit of screening a significantly higher number of substances per unit of time for their influence on the response. For example, if the single experiment performed on the microscope can be run in at least 96 experimental chambers simultaneously the throughput for the person who is running the experiments increases by a factor of 96.

Imaging plate readers integrate the signal from each well into a single value per time point. Thus the data resulting from a single "run" of the instrument is a set of time series of single values, one for each well, with the injection of the test compound taking place at a known point in the time series. The primary advantage of this type of instrumentation is that it greatly increases the number of samples that can be processed in a given amount of time (the throughput). This is of great advantage when using the assay in a screening program for new pharmaceutical lead compounds.

The first step in the data analysis is to normalise the results from each well so that they can be compared with each other or with previously analysed known compounds. This

always begins with correcting the signal by subtracting the instrument bias from all data points on a well-by-well basis. From this point, either of two techniques can be followed depending on the design of the assay:

Procedure 1: The average of the signal prior to the addition of the test compound is subtracted from all data points on a well-by-well basis.

Procedure 2: The data are corrected for any known background by subtracting the background value from all data points on a well-by-well basis. The resulting background-corrected data are normalised by dividing each data set by the average of the data values prior to the injection of the test compound on a well-by-well basis.

The corrected or normalised time series data sets are then further reduced by a technique that converts the time series to a single value. There are at least three such approaches:

For transient responses, the maximum deviation from the baseline is determined. This is also known as the "peak height" technique.

Alternatively, the signal is integrated over time between pre-defined limits. If the data were treated according to Procedure 2 above, then the offset is subtracted such that the integral of a non-response is zero within the limit of measurement error. This is also known as the "peak area" technique. If the response is a cumulative one, e.g., an exponential change to a new level, the result is taken as the either the difference or the ratio between the signal after a predetermined time and the signal prior to the addition of the test compound.

All of the above procedures reduce the data for a given well to one or more single values. For screening purposes, these values will be searched for those that are greater than a certain statistically determined cut-off value. For characterisation, the values represent a quantitative response, and are further treated in sets by techniques such as dose-response curve fitting.

In another embodiment of the invention, the measurement of redistribution is accomplished indirectly by taking advantage of the fact that in order for redistribution to occur, the probe will experience some change in its freedom, or restriction, of movement within the intracellular milieu. The degree of translocation will correlate with the amount of freely mobile luminophore in the cytoplasm. At a point in time after the test compound

has begun to have any influence it may have, the amount or fraction of restricted luminophore can be measured by disrupting or permeabilising the plasma membrane of the cells and allowing the freely mobile luminophore to diffuse away. If the detection volume of the detector is limited to the region immediately surrounding the cells, and the overall volume into which the freely mobile luminophore can diffuse is much larger, then the freely mobile luminophore essentially disappears from the detector's view and its signal is not recorded.

In one aspect of the invention, the above mentioned measurement of redistribution is made on cells with permanently permeabilised plasma membranes immersed in a solution mimicking the cytoplasmic environment. In this way the influence of compounds that can normally not enter the cytoplasm of cells can be tested.

The nucleic acid constructs used in the present invention encode in their nucleic acid sequences fusion polypeptides comprising a biologically active polypeptide that is a component of an intracellular signalling pathway, or a part thereof, and a GFP, preferably an F64L mutant of GFP, N- or C-terminally fused, optionally via a peptide linker, to the biologically active polypeptide or part thereof. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein kinase or a phosphatase. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a transcription factor or a part thereof which changes cellular localisation upon activation.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein, or a part thereof, which is associated with the cytoskeletal network and which changes cellular localisation upon activation. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein kinase or a part thereof which changes cellular localisation upon activation. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a serine/threonine protein kinase or a part thereof capable of changing intracellular localisation upon activation. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a tyrosine protein kinase or a part thereof capable of changing intracellular localisation upon activation. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a phospholipid-

dependent serine/threonine protein kinase or a part thereof capable of changing intracellular localisation upon activation.

In a specific embodiment the constructs listed in table 1 are used in a method for extracting quantitative information relating to an influence on a cellular response in mechanically intact or permeablised living cells, the method comprising recording variation in spatially distributed fluorescence emitted from the fluorophore being present in the cells and being capable of being redistributed in a manner which is related with the degree of the influence, and/or being modulated by a component which is capable of being redistributed in a manner which is related to the degree of the influence, as a change fluorescence intensity preferably measured by an instrument designed for the measurement of changes in fluorescence intensity.

Table 1 The fusion constructs of the invention by the names used herein as well as by reference to relevant SEQ ID NOs of sequences of DNA encoding the construct and full amino acid sequences.

Fusion construct	DNA sequence SEQ ID NO:	Protein Sequence SEQ ID NO:
PKAcat - F64LS65TGFP	1	2
PKCα- F64L-S65TGFP	3	4
EGFP - Erk1	5	6
EGFP - SMAD2	7	8
SMAD2 - EGFP	9	10
EGFP - VASP	11	12
EGFP - NFχβ	13	14
NFχβ - EGFP	15	16
EGFP - PKCβ1	17	18

As illustrated in examples 8, 9 and 11, the redistribution of PKA, and PKC can readily be detected as a variation in fluorescence intensity, as measured e.g. in the FLIPR™ instrument.

In one embodiment any new luminophore determined to redistribute in response to an influence in a pattern similar to the pattern observed in the microscope for PKA or PKC (see examples 1, 2, 8 and 11), that is from an aggregated form to a dispersed form or from a dispersed form to an aggregated form of the luminophore as the redistribution takes place, can be predicted to be detectable as a variation in light intensity as measured, for example in the FLIPR™ instrument.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a cAMP-dependent protein kinase or a part thereof capable of changing cellular localisation upon activation. In a preferred embodiment the biologically active polypeptide encoded by the nucleic acid construct is a PKAc-F64L-S65T-GFP fusion. In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a cGMP-dependent protein kinase or a part thereof capable of changing cellular localisation upon activation.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a calmodulin-dependent serine/threonine protein kinase or a part thereof capable of changing cellular localisation upon activation.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a mitogen-activated serine/threonine protein kinase or a part thereof capable of changing cellular localisation upon activation. In preferred embodiments the biologically active polypeptide encoded by the nucleic acid constructs are an ERK1-F64L-S65T-GFP fusion or an EGFP-ERK1 fusion.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a cyclin-dependent serine/threonine protein kinase or a part thereof capable of changing cellular localisation upon activation.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct is a protein phosphatase or a part thereof capable of changing cellular localisation upon activation.

In one preferred embodiment of the invention the nucleic acid constructs may be DNA constructs.

In one embodiment the biologically active polypeptide encoded by the nucleic acid construct. In one embodiment the gene encoding GFP in the nucleic acid construct is derived from Aequorea victoria. In a preferred embodiment the gene encoding GFP in the nucleic acid construct is EGFP or a GFP variant selected from F64L-GFP, F64L-Y66H-GFP and F64L-S65T-GFP.

In preferred embodiments of the invention the DNA constructs which can be identified by any of the DNA sequences shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15 and 17 or are variants of these sequences capable of encoding the same fusion polypeptide or a fusion polypeptide which is biologically equivalent thereto, e.g. an isoform, or a splice variant or a homologue from another species.

The present invention describes a method that may be used to establish a screening program for the identification of biologically active substances that directly or indirectly affects intracellular signalling pathways and because of this property are potentially useful as medicaments. Based on measurements in living cells of the redistribution of spatially resolved luminescence from luminophores which undergo a change in distribution upon activation or deactivation of an intracellular signalling pathway the result of the individual measurement of each substance being screened indicates its potential biological activity.

In one embodiment of the invention the screening program is used for the identification of a biologically toxic substance as defined herein that exerts its toxic effect by interfering with an intracellular signalling pathway. Based on measurements in living cells of the redistribution of spatially resolved luminescence from luminophores which undergo a change in distribution upon activation or deactivation of an intracellular signalling pathway the result of the individual measurement of each substance being screened indicates its potential biologically toxic activity. In one embodiment of a screening program a compound that modulates a component of an intracellular pathway as defined herein, can be found and the therapeutic amount of the compound estimated by a method according to the method of the invention. In a preferred embodiment the present invention leads to the discovery of a new way of treating a condition or disease related to the intracellular function of a biologically active polypeptide comprising administration to a patient suffering from said condition or disease of an effective amount of a compound which has been discovered by any method according to the invention. In another preferred embodiment of the invention a method is established for identification of a new drug target or several new drug targets among the group of biologically active polypeptides which are components of intracellular signalling pathways.

In another embodiment of the invention an individual treatment regimen is established for the selective treatment of a selected patient suffering from an ailment where the available medicaments used for treatment of the ailment are tested on a relevant primary cell or cells obtained from said patient from one or several tissues, using a method comprising transfecting the cell or cells with at least one DNA sequence encoding a fluorescent probe according to the invention, transferring the transfected cell or cells back the said patient, or culturing the cell or cells under conditions permitting the expression of said probes and exposing it to an array of the available medicaments, then comparing changes in fluorescence patterns or redistribution patterns of the fluorescent probes in the intact living cells to detect the cellular response to the specific medicaments (obtaining a cellular action profile), then selecting one or more medicament or medicaments based on the desired activity and acceptable level of side effects and administering an effective amount of these medicaments to the selected patient.

The present invention describes a method that may be used to establish a screening program for back-tracking signal transduction pathways as defined herein. In one embodiment the screening program is used to establish more precisely at which level one or several compounds affect a specific signal transduction pathway by successively or in parallel testing the influence of the compound or compounds on the redistribution of spatially resolved luminescence from several of the luminophores which undergo a change in distribution upon activation or deactivation of the intracellular signalling pathway under study.

In general, a probe, i.e. a "GeneX"-GFP fusion or a GFP-"GeneX" fusion, is constructed using PCR with "GeneX"-specific primers followed by a cloning step to fuse "GeneX" in frame with GFP. The fusion may contain a short vector derived sequence between "GeneX" and GFP (e.g. part of a multiple cloning site region in the plasmid) resulting in a peptide linker between "GeneX" and GFP in the resulting fusion protein.

Some of the steps involved in the development of a probe include the following: Identify the sequence of the gene. This is most readily done by searching a depository of genetic information, e.g. the GenBank Sequence Database, which is widely available and routinely used by molecular biologists. In the specific examples below the GenBank Accession number of the gene in question is provided.

Design the gene-specific primers. Inspection of the sequence of the gene allows design of gene-specific primers to be used in a PCR reaction. Typically, the top-strand primer encompasses the ATG start codon of the gene and the following ca. 20 nucleotides, while the bottom-strand primer encompasses the stop codon and the ca. 20 preceding nucleotides, if the gene is to be fused behind GFP, i.e. a GFP-"GeneX" fusion. If the gene is to be fused in front of GFP, i.e. a "GeneX"-GFP fusion, a stop codon must be avoided. Optionally, the full-length sequence of GeneX may not be used in the fusion, but merely the part that localizes and redistributes like GeneX in response to a signal. In addition to gene-specific sequences, the primers contain at least one recognition sequence for a restriction enzyme, to allow subsequent cloning of the PCR product. The sites are chosen so that they are unique in the PCR product and compatible with sites in the cloning vector. Furthermore, it may be necessary to include an exact number of nucleotides between the restriction enzyme site and the gene-specific sequence in order to establish the correct reading frame of the fusion gene and/or a translation initiation consensus sequence. Lastly, the primers always contain a few nucleotides in front of the restriction enzyme site to allow efficient digestion with the enzyme.

Identify a source of the gene to be amplified. In order for a PCR reaction to produce a product with gene-specific primers, the gene-sequence must initially be present in the reaction, e.g. in the form of cDNA. Information in GenBank or the scientific literature will usually indicate in which tissue(s) the gene is expressed, and cDNA libraries from a great variety of tissues or cell types from various species are commercially available, e.g. from Clontech (Palo Alto), Stratagene (La Jolla) and Invitrogen (San Diego). Many genes are also available in cloned form from The American Type Tissue Collection (Virginia).

Optimise the PCR reaction. Several factors are known to influence the efficiency and specificity of a PCR reaction, including the annealing temperature of the primers, the concentration of ions, notably Mg^{2+} and K^{+}, present in the reaction, as well as pH of the reaction. If the result of a PCR reaction is deemed unsatisfactory, it might be because the parameters mentioned above are not optimal. Various annealing temperatures should be tested, e.g. in a PCR machine with a built-in temperature gradient, available from e.g. Stratagene (La Jolla), and/or various buffer compositions should be tried, e.g. the OptiPrime buffer system from Stratagene (La Jolla).

Clone the PCR product. The vector into which the amplified gene product will be cloned and fused with GFP will already have been taken into consideration when the primers

were designed. When choosing a vector, one should at least consider in which cell types the probe subsequently will be expressed, so that the promoter controlling expression of the probe is compatible with the cells. Most expression vectors also contain one or more selective markers, e.g. conferring resistance to a drug, which is a useful feature when one wants to make stable transfectants. The selective marker should also be compatible with the cells to be used.

The actual cloning of the PCR product should present no difficulty as it typically will be a one-step cloning of a fragment digested with two different restriction enzymes into a vector digested with the same two enzymes. If the cloning proves to be problematic, it may be because the restriction enzymes did not work well with the PCR fragment. In this case one could add longer extensions to the end of the primers to overcome a possible difficulty of digestion close to a fragment end, or one could introduce an intermediate cloning step not based on restriction enzyme digestion. Several companies offer systems for this approach, e.g. Invitrogen (San Diego) and Clontech (Palo Alto).

Once the gene has been cloned and, in the process, fused with the GFP gene, the resulting product, usually a plasmid, should be carefully checked to make sure it is as expected. The most exact test would be to obtain the nucleotide sequence of the fusion-gene.

Once a DNA construct for a probe has been generated, its functionality and usefulness may be evaluated by transfecting it into cells capable of expressing the probe. The fluorescence of the cell is inspected soon after, typically the next day. At this point, two features of cellular fluorescence are noted: the intensity and the sub-cellular localisation.

The intensity should usually be at least as strong as that of unfused GFP in the cells. If it is not, the sequence or quality of the probe-DNA might be faulty, and should be carefully checked.

The sub-cellular localisation is an indication of whether the probe is likely to perform well. If it localises as expected for the gene in question, e.g. is excluded from the nucleus, it can immediately go on to a functional test. If the probe is not localised soon after the transfection procedure, it may be because of overexpression at this point in time, as the cell typically will have taken up very many copies of the plasmid, and localisation will

occur in time, e.g. within a few weeks, as plasmid copy number and expression level decreases. If localisation does not occur after prolonged time, it may be because the fusion to GFP has destroyed a localisation function, e.g. masked a protein sequence essential for interaction with its normal cellular anchor-protein. In this case the opposite fusion might work, e.g. if GeneX-GFP does not work, GFP-GeneX might, as two different parts of GeneX will be affected by the proximity to GFP. If this does not work, the proximity of GFP at either end might be a problem, and it could be attempted to increase the distance by incorporating a longer linker between GeneX and GFP in the DNA construct.

If there is no prior knowledge of localisation, and no localisation is observed, it may be because the probe should not be localised at this point, because such is the nature of the protein fused to GFP. It should then be subjected to a functional test.

In a functional test, the cells expressing the probe are treated with at least one compound known to perturb, usually by activating, the signalling pathway on which the probe is expected to report by redistributing itself within the cell. If the redistribution is as expected, e.g. if prior knowledge tell that it should translocate from location X to location Y, it has passed the first critical test. In this case it can go on to further characterisation and quantification of the response.

If it does not perform as expected, it may be because the cell lacks at least one component of the signalling pathway, e.g. a cell surface receptor, or there is species incompatibility, e.g. if the probe is modelled on sequence information of a human gene product, and the cell is of hamster origin. In both instances one should identify other cell types for the testing process where these potential problems would not apply.

If there is no prior knowledge about the pattern of redistribution, the analysis of the redistribution will have to be done in greater depth to identify what the essential and indicative features are, and when this is clear, it can go on to further characterisation and quantification of the response. If no feature of redistribution can be identified, the problem might be as mentioned above, and the probe should be retested under more optimal cellular conditions. If the probe does not perform under optimal cellular conditions, then it's back to the drawing board.

The process of developing an image-based redistribution assay begins with either the unplanned experimental observation that a redistribution phenomenon can be visualised, or the design of a probe specifically to follow a redistribution phenomenon already known to occur. In either event, the first and best exploratory technique is for a trained scientist or technician to observe the phenomenon. Even with the rapid advances in computing technology, the human eye-brain combination is still the most powerful pattern recognition system known, and requires no advance knowledge of the system in order to detect potentially interesting and useful patterns in raw data. This is especially if those data are presented in the form of images, which are the natural "data type" for human visual processing. Because human visual processing operates most effectively in a relatively narrow frequency range, i.e., we cannot see either very fast or very slow changes in our visual field, it may be necessary to record the data and play it back with either time dilation or time compression.

Some luminescence phenomena cannot be seen directly by the human eye. Examples include polarisation and fluorescence lifetime. However, with suitable filters or detectors, these signals can be recorded as images or sequences of images and displayed to the human in the fashion just described. In this way, patterns can be detected and the same methods can be applied.

Once the redistribution has been determined to be a reproducible phenomenon, one or more data sets are generated for the purpose of developing a procedure for extracting the quantitative information from the data. In parallel, the biological and optical conditions are determined which will give the best quality raw data for the assay. This can become an iterative process; it may be necessary to develop a quantitative procedure in order to assess the effect on the assay of manipulating the assay conditions.

The data sets are examined by a person or persons with knowledge of the biological phenomenon and skill in the application of image processing techniques. The goal of this exercise is to determine or at least propose a method that will reduce the image or sequence of images constituting the record of a "response" to a value corresponding to the degree of the response. Using either interactive image processing software or an image processing toolbox and a programming language, the method is encoded as a procedure or algorithm that takes the image or images as input and generates the

degree of response (in any units) as its output. Some of the criteria for evaluating the validity of a particular procedure are:

Does the degree of the response vary in a biologically significant fashion, i.e., does it show the known or putative dependence on the concentration of the stimulating agent or condition?

Is the degree of response reproducible, i.e., does the same concentration or level of stimulating agent or condition give the same response with an acceptable variance? Is the dynamic range of the response sufficient for the purpose of the assay? If not, can a change in the procedure or one of its parameters improve the dynamic range? Does the procedure exhibit any clear "pathologies", i.e., does it give ridiculous values for the response if there are commonly occurring imperfections in the imaging process? Can these pathologies be eliminated, controlled, or accounted for? Can the procedure deal with the normal variation in the number and/or size of cells in an image?

In some cases the method may be obvious; in others, a number of possible procedures may suggest themselves. Even if one method appears clearly superior to others, optimisation of parameters may be required. The various procedures are applied to the data set and the criteria suggested above are determined, or the single procedure is applied repeatedly with adjustment of the parameter or parameters until the most satisfactory combination of signal, noise, range, etc. are arrived at. This is equivalent to the calibration of any type of single-channel sensor.

The number of ways of extracting a single value from an image are extremely large, and thus an intelligent approach must be taken to the initial step of reducing this number to a small, finite number of possible procedures. This is not to say that the procedure arrived at is necessarily the best procedure - but a global search for the best procedure is simply out of the question due to the sheer number of possibilities involved.

Image-based assays are no different than other assay techniques in that their usefulness is characterised by parameters such as the specificity for the desired component of the sample, the dynamic range, the variance, the sensitivity, the concentration range over which the assay will work, and other such parameters. While it is not necessary to

characterise each and every one of these before using the assay, they represent the only way to compare one assay with another.

The final step is then to see whether there exists a possibility to increase the throughput of the assay to improve its utility as the basis of a screening program. In order to do this, a dose-response relationship is established based on quantification of the responses caused by a particular influence, representative of the underlying intracellular signalling process, using the methods described above and in the examples. The dose-response relationship for the particular influence is then compared to the dose-response relationship obtained by performing the same assay in an instrument which allows parallel monitoring of all wells in a microtiter plate such as a FLIPR™ or an ordinary imaging or fluorescence plate reader for microtiter plates. If a good correlation between the dose-response relationships obtained from the two different measurement systems is obtained, it can be said that the parallel measurement mode has been validated (see examples 8, 9 and 11). This implies that it can be used as the primary basis for a screening program with the potential benefit of screening a significantly higher number of substances for their influence on the response per unit of time.

In the present specification and claims, the term "an influence" covers any influence to which the cellular response comprises a redistribution. Thus, e.g., heating, cooling, pH, high pressure, low pressure, humidifying, or drying are influences on the cellular response on which the resulting redistribution can be quantified, but as mentioned above, perhaps the most important influences are the influences of contacting or incubating the cells with substances which are known or suspected to exert an influence on the cellular response involving a redistribution contribution. In another embodiment of the invention the influence could be substances from a compound drug library.

In the present context, the term "green fluorescent protein" is intended to indicate a protein which, when expressed by a cell, emits fluorescence upon exposure to light of the correct excitation wavelength (cf. [(Chalfie, M. *et al.* (1994) Science 263, 802-805)]). In the following, GFP in which one or more amino acids have been substituted, inserted or deleted is most often termed "modified GFP". "GFP" as used herein includes wild-type GFP derived from the jelly fish *Aequorea victoria* and modifications of GFP, such as the blue fluorescent variant of GFP disclosed by Heim *et al.* (1994). Proc.Natl.Acad.Sci. 91:26, pp 12501-12504, and other modifications that change the spectral properties of the GFP

fluorescence, or modifications that exhibit increased fluorescence when expressed in cells at a temperature above about 30°C described in PCT/DK96/00051, published as WO 97/11094 on 27 March 1997 and hereby incorporated by reference, and which comprises a fluorescent protein derived from *Aequorea* Green Fluorescent Protein (GFP) or any functional analogue thereof, wherein the amino acid in position 1 upstream from the chromophore has been mutated to provide an increase of fluorescence intensity when the fluorescent protein of the invention is expressed in cells. Preferred GFP variants are F64L-GFP, F64L-Y66H-GFP and F64L-S65T-GFP. An especially preferred variant of GFP for use in all the aspects of this invention is EGFP (DNA encoding EGFP which is a F64L-S65T variant with codons optimized for expression in mammalian cells is available from Clontech, Palo Alto, plasmids containing the EGFP DNA sequence, cf. GenBank Acc. Nos. U55762, U55763).

The term "intracellular signalling pathway" and "signal transduction pathway" are intended to indicate the co-ordinated intracellular processes whereby a living cell transduce an external or internal signal into cellular responses. Said signal transduction will involve an enzymatic reaction said enzymes include but are not limited to protein kinases, GTPases, ATPases, protein phosphatases, phospholipases and cyclic nucleotide phosphodiesterases. The cellular responses include but are not limited to gene transcription, secretion, proliferation, mechanical activity, metabolic activity, cell death.

The term "second messenger" is used to indicate a low molecular weight component involved in the early events of intracellular signal transduction pathways.

The term "luminophore" is used to indicate a chemical substance that has the property of emitting light either inherently or upon stimulation with chemical or physical means. This includes but is not limited to fluorescence, bioluminescence. phosphorescence, and chemiluminescence.

The term "mechanically intact living cell" is used to indicate a cell which is considered living according to standard criteria for that particular type of cell such as maintenance of normal membrane potential, energy metabolism, proliferative capability, and has not experienced any physically invasive treatment designed to introduce external substances into the cell such as microinjection.

In the present context, the term "permeabilised living cell" is used to indicate cells where a pore forming agent such as Streptolysin O or *Staphylococcus Aureus* α-toxin has been applied and thereby incorporated into the plasma membrane in the cells. This creates proteinaceous pores with a defined pore size in the plasma membranes of the exposed cells. Pores could also be made by electroporation, i.e. exposing the cells to high voltage discharges, a procedure that creates small holes in the plasma membrane by coagulating integral membrane proteins. Treatment with a mild detergent such as saponin may accomplish the same thing. Common to all these treatments are that pores are formed only in the plasma membrane without affecting the integrity of cytoplasmic structural elements and organelles. The term living in this context means that the permeabilised cells bathed in a solution mimicking the intracellular milieu still have functional organelles, such as actively respiring mitochondria and endoplasmic reticulum that can take up and release calcium ions, and functional structural elements. The benefit of this method is that substances that normally can not traverse the plasma membrane, but most likely exert their influence intracellularly, can be introduced and their influence studied without cumbersome microinjection of the substances into single cells. Using this method the response to an influence can be recorded from many cells simultaneously.

In the present context, the term "permeabilisation" is intended to indicate the selective disruption of the plasma membrane barrier so that soluble substances freely mobile in the cytosol are lost from the cells. The permeabilisation can be achieved as described above under "permeabilised living cells" or by using other chemical detergents such as Triton X-100 or digitonin in carefully titrated amounts.

The term "physiologically relevant", when applied to an experimentally determined redistribution of an intracellular component, as measured by a change in the luminescence properties or distribution, is used to indicate that said redistribution can be explained in terms of the underlying biological phenomenon which gives rise to the redistribution.

The terms "image processing" and "image analysis" are used to describe a large family of digital data analysis techniques or combination of such techniques which reduce ordered arrays of numbers (images) to quantitative information describing those ordered

arrays of numbers. When said ordered arrays of numbers represent measured values from a physical process, the quantitative information derived is therefore a measure of the physical process.

The term "fluorescent probe" is used to indicate a fluorescent fusion polypeptide comprising a GFP or any functional part thereof which is N- or C-terminally fused to a biologically active polypeptide as defined herein, optionally via a peptide linker consisting of one or more amino acid residues, where the size of the linker peptide in itself is not critical as long as the desired functionality of the fluorescent probe is maintained. A fluorescent probe according to the invention is expressed in a cell and basically mimics the physiological behaviour of the biologically active polypeptide moiety of the fusion polypeptide.

The term "mammalian cell" is intended to indicate any living cell of mammalian origin. The cell may be an established cell line, many of which are available from The American Type Culture Collection (ATCC, Virginia, USA) or a primary cell with a limited life span derived from a mammalian tissue. including tissues derived from a transgenic animal, or a newly established immortal cell line derived from a mammalian tissue including transgenic tissues, or a hybrid cell or cell line derived by fusing different cell types of mammalian origin e.g. hybridoma cell lines. The cells may optionally express one or more non-native gene products, e.g. receptors, enzymes, enzyme substrates, prior to or in addition to the fluorescent probe. Preferred cell lines include but are not limited to those of fibroblast origin, e.g. BHK, CHO, BALB, or of endothelial origin, e.g. HUVEC, BAE (bovine artery endothelial), CPAE (cow pulmonary artery endothelial), HLMVEC (human lung microvascular endothelial cells) or of pancreatic origin, e.g. RIN, INS-1, MIN6, bTC3, aTC6, bTC6, HIT, or of hematopoietic origin, e.g.primary isolated human monocytes, macrophages, neutrophils, basophils, eosinophils and lyphocyte populations, AML-193, HL-60, RBL-1, adipocyte origin, e.g. 3T3-L1, neuronal/neuroendocrine origin, e.g. AtT20, PC12, GH3, muscle origin, e.g. SKMC, A10, C2C12, renal origin, e.g. HEK 293, LLC-PK1.

The term "hybrid polypeptide" is intended to indicate a polypeptide which is a fusion of at least a portion of each of two proteins, in this case at least a portion of the green fluorescent protein, and at least a portion of a catalytic and/or regulatory domain of a protein kinase. Furthermore a hybrid polypeptide is intended to indicate a fusion

polypeptide comprising a GFP or at least a portion of the green fluorescent protein that contains a functional fluorophore, and at least a portion of a biologically active polypeptide. Thus, GFP may be N- or C-terminally tagged to a biologically active polypeptide, optionally via a linker portion or linker peptide consisting of a sequence of one or more amino acids. The hybrid polypeptide or fusion polypeptide may act as a fluorescent probe in intact living cells carrying a DNA sequence encoding the hybrid polypeptide under conditions permitting expression of said hybrid polypeptide.

The term "kinase" is intended to indicate an enzyme that is capable of phosphorylating a cellular component.

The term "protein kinase" is intended to indicate an enzyme that is capable of phosphorylating serine and/or threonine and/or tyrosine in peptides and/or proteins.

The term "phosphatase" is intended to indicate an enzyme that is capable of dephosphorylating phosphoserine and/or phosphothreonine and/or phosphotyrosine in peptides and/or proteins.

The term "cyclic nucleotide phosphodiesterase" is intended to indicate an enzyme that is capable of inactivating the second messengers cAMP and cGMP by hydrolysis of their 3'-ester bond.

In the present context, the term "biologically active polypeptide" is intended to indicate a polypeptide affecting intracellular processes upon activation, such as an enzyme which is active in intracellular processes or a portion thereof comprising a desired amino acid sequence which has a biological function or exerts a biological effect in a cellular system. In the polypeptide one or several amino acids may have been deleted, inserted or replaced to alter its biological function, e.g. by rendering a catalytic site inactive. Preferably, the biologically active polypeptide is selected from the group consisting of proteins taking part in an intracellular signalling pathway, such as enzymes involved in the intracellular phosphorylation and dephosphorylation processes including kinases, protein kinases and phosphorylases as defined herein, but also proteins making up the cytoskeleton play important roles in intracellular signal transduction and are therefore included in the meaning of "biologically active polypeptide" herein. More preferably, the biologically active polypeptide is a protein which according to its state as activated or

non-activated changes localisation within the cell, preferably as an intermediary component in a signal transduction pathway. Included in this preferred group of biologically active polypeptides are cAMP dependent protein kinase A.

The term "a substance having biological activity" is intended to indicate any sample that has a biological function or exerts a biological effect in a cellular system. The sample may be a sample of a biological material such as a sample of a body fluid including blood, plasma, saliva, milk, urine, or a microbial or plant extract, an environmental sample containing pollutants including heavy metals or toxins, or it may be a sample containing a compound or mixture of compounds prepared by organic synthesis or genetic techniques.

The phrase "any change in fluorescence" means any change in absorption properties, such as wavelength and intensity, or any change in spectral properties of the emitted light, such as a change of wavelength, fluorescence lifetime, intensity or polarisation, or any change in the intracellular localisation of the fluorophore. It may thus be localised to a specific cellular component (e.g. organelle, membrane, cytoskeleton, molecular structure) or it may be evenly distributed throughout the cell or parts of the cell.

The term "organism" as used herein indicates any unicellular or multicellular organism preferably originating from the animal kingdom including protozoans, but also organisms that are members of the plant kingdoms, such as algae, fungi. bryophytes, and vascular plants are included in this definition.

The term "nucleic acid" is intended to indicate any type of poly- or oligonucleic acid sequence, such as a DNA sequence, a cDNA sequence, or an RNA sequence.

The term "biologically equivalent" as it relates to proteins is intended to mean that a first protein is equivalent to a second protein if the cellular functions of the two proteins may substitute for each other, e.g. if the two proteins are closely related isoforms encoded by different genes, if they are splicing variants, or allelic variants derived from the same gene, if they perform identical cellular functions in different cell types, or in different species. The term "biologically equivalent" as it relates to DNA is intended to mean that a first DNA sequence encoding a polypeptide is equivalent to a second DNA sequence

encoding a polypeptide if the functional proteins encoded by the two genes are biologically equivalent.

The term "higher throughput" is intended to mean an increased number of experiments per time unit per person performing the actual experiments.

The term "high throughput screening assay" as used herein is intended to mean the process of performing a screening assay with at least 100 individual experiments where compounds are tested for their influence on the redistribution of a luminophore in one working day for one person skilled in the art. In a preferred embodiment the high throughput screening assay involves at least 500 individual experiments such as 750, 1000, 2000, 5000, or even 10.000 individual experiments in one working day for a person skilled in the art.

The phrase "back-tracking of a signal transduction pathway" is intended to indicate a process for defining more precisely at what level a signal transduction pathway is affected, either by the influence of chemical compounds or a disease state in an organism. Consider a specific signal transduction pathway represented by the bioactive polypeptides A - B - C - D, with signal transduction from A towards D. When investigating all components of this signal transduction pathway compounds or disease states that influence the activity or redistribution of only D can be considered to act on C or downstream of C whereas compounds or disease states that influence the activity or redistribution of C and D, but not of A and B can be considered to act downstream of B.

The term "fixed cells" is used to mean cells treated with a cytological fixative such as glutaraldehyde or formaldehyde, treatments that serve to chemically cross-link and stabilise soluble and insoluble proteins within the structure of the cell. Once in this state, such proteins cannot be lost from the structure of the now-dead cell.

In the present context a "screening assay" is intended to mean any measurement protocol, including materials, cells. instruments, chemicals, reagents, detection units, calibration and quantification procedures used to measure a response from mechanically intact or permeabilised living cells relevant to influences on an intracellular pathway.

The term "dose-response relationship" and "screening programme" is in the present context intended to mean a clear correlation between the quantified response of cells in a screening assay to application of an influence, such as a compound, and the concentration of the applied influence. The response to the influence may be both an up-regulation and a down-regulation of the quantified parameter used in the screening assay.

In the present context, the term "physiology" is intended to mean the normal function of biological and biochemical processes inside cells, between cells and in the whole organism or animal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. CHO cells expressing the PKAc-F64L-S65T-GFP hybrid protein have been treated in HAM's F12 medium with 50 μM forskolin at 37°C. The images of the GFP fluorescence in these cells have been taken at different time intervals after treatment, which were: a) 40 seconds b) 60 seconds c) 70 seconds d) 80 seconds. The fluorescence changes from a punctate to a more even distribution within the (non-nuclear) cytoplasm.

Figure 2. Time-lapse analysis of forskolin induced PKAc-F64L-S65T-GFP redistribution. CHO cells, expressing the PKAc-F64L-S65T-GFP fusion protein were analysed by time-lapse fluorescence microscopy. Fluorescence micrographs were acquired at regular intervals from 2 min before to 8 min after the addition of agonist. The cells were challenged with 1 μM forskolin immediately after the upper left image was acquired (t=0). Frames were collected at the following times: i) 0, ii) 1, iii) 2, iv) 3, v) 4 and vi) 5 minutes. Scale bar 10 μm.

Figure 3. Time-lapse analyses of PKAc-F64L-S65T-GFP redistribution in response to various agonists. The effects of 1 μM forskolin (A), 50 μM forskolin (B), 1mM dbcAMP (C) and 100 μM IBMX (D) (additions indicated by open arrows) on the localisation of the PKAc-F64L-S65T-GFP fusion protein were analysed by time-lapse fluorescence microscopy of CHO/PKAc-F64L-S65T-GFP cells. The effect of addition of 10 μM forskolin (open arrow), followed shortly by repeated washing with buffer (solid arrow), on the localisation of the PKAc-F64L-S65T-GFP fusion protein was analysed in the same

cells (E). In a parallel experiment, the effect of adding 10 μM forskolin and 100 μM IBMX (open arrow) followed by repeated washing with buffer containing 100 μM IBMX (solid arrow) was analysed (F). Removing forskolin caused PKAc-F64L-S65T-GFP fusion protein to return to the cytoplasmic aggregates while this is prevented by the continued presence of IBMX (F). The effect of 100 nM glucagon (Fig 3G, open arrow) on the localisation of the PKAc-F64L-S65T-GFP fusion protein is also shown for BHK/GR, PKAc-F64L-S65T-GFP cells. The effect of 10 μM norepinephrine (H), solid arrow, on the localisation of the PKAc-F64L-S65T-GFP fusion protein was analysed similarly, in transiently transfected CHO, PKAc-F64L-S65T-GFP cells, pretreated with 10 μM forskolin, open arrow, to increase [cAMP]. N.B. in Fig 3H the x-axis counts the image numbers, with 12 seconds between images. The raw data of each experiment consisted of 60 fluorescence micrographs acquired at regular intervals including several images acquired before the addition of buffer or agonist. The charts (A-G) each show a quantification of the response seen through all the 60 images, performed as described in analysis method 2. The change in total area of the highly fluorescent aggregates, relative to the initial area of fluorescent aggregates is plotted as the ordinate in all graphs in Figure 3, versus time for each experiment. Scale bar 10 μm.

Figure 4. Dose-response curve (two experiments) for forskolin-induced redistribution of the PKAc-F64L-S65T-GFP fusion.

Figure 5. Time from initiation of a response to half maximal ($t_{1/2max}$) and maximal (t_{max}) PKAc-F64L-S65T-GFP redistribution. The data was extracted from curves such as that shown in "Figure 2." All $t_{1/2max}$ and t_{max} values are given as mean±SD and are based on a total of 26-30 cells from 2-3 independent experiments for each forskolin concentration. Since the observed redistribution is sustained over time, the t_{max} values were taken as the earliest time point at which complete redistribution is reached. Note that the values do not relate to the degree of redistribution.

Figure 6. Parallel dose-response analyses of forskolin induced cAMP elevation and PKAc-F64L-S65T-GFP redistribution. The effects of buffer or 5 increasing concentrations of forskolin on the localisation of the PKAc-F64L-S65T-GFP fusion protein in CHO/PKAc-F64L-S65T-GFP cells, grown in a 96 well plate, were analysed as described above. Computing the ratio of the SD's of fluorescence micrographs taken of the same field of cells, prior to and 30 min after the addition of forskolin, gave a reproducible measure of

PKAc-F64L-S65T-GFP redistribution. The graph shows the individual 48 measurements and a trace of their mean±s.e.m at each forskolin concentration. For comparison, the effects of buffer or 8 increasing concentrations of forskolin on $[cAMP]_i$ was analysed by a scintillation proximity assay of cells grown under the same conditions. The graph shows a trace of the mean ± s.e.m of 4 experiments expressed in arbitrary units.

Figure 7. BHK cells stably transfected with the human muscarinic (hM1) receptor and the PKCα-F64L-S65T-GFP fusion. Carbachol (100 μM added at 1.0 second) induced a transient redistribution of PKCα-F64L-S65T-GFP from the cytoplasm to the plasma membrane. Images were taken at the following times: a) 1 second before carbachol addition, b) 8.8 seconds after addition and c) 52.8 seconds after addition.

Figure 8. BHK cells stably transfected with the hM1 receptor and PKCα-F64L-S65T-GFP fusion were treated with carbachol (1 μM, 10 μM, 100 μM). In single cells intracellular $[Ca^{2+}]$ was monitored simultaneously with the redistribution of PKCα-F64L-S65T-GFP. Dashed line indicates the addition times of carbachol. The top panel shows changes in the intracellular Ca^{2+} concentration of individual cells with time for each treatment. The middle panel shows changes in the average cytoplasmic GFP fluorescence for individual cells against time for each treatment. The bottom panel shows changes in the fluorescence of the periphery of single cells, within regions that specifically include the circumferential edge of a cell as seen in normal projection, the best regions for monitoring changes in the fluorescence intensity of the plasma membrane.

Figure 9. The hERK1-F64L-S65T-GFP fusion expressed in HEK293 cells treated with 100 μM of the MEK1 inhibitor PD98059 in HAM F-12 (without serum) for 30 minutes at 37 °C. The nuclei empty of fluorescence during this treatment. The same cells as in (a) following treatment with 10 % foetal calf serum for 15 minutes at 37 °C.
Time profiles for the redistribution of GFP fluorescence in HEK293 cells following treatment with various concentrations of EGF in Hepes buffer (HAM F-12 replaced with Hepes buffer directly before the experiment). Redistribution of fluorescence is expressed as the change in the ratio value between areas in nucleus and cytoplasm of single cells. Each time profile is the mean for the changes seen in six single cells.
Bar chart for the end-point measurements, 600 seconds after start of EGF treatments, of fluorescence change (nucleus:cytoplasm) following various concentrations of EGF.

Figure 10. The SMAD2-EGFP fusion expressed in HEK293 cells starved of serum overnight in HAM F-12. HAM F-12 was then replaced with Hepes buffer pH 7.2 immediately before the experiment. Scale bar is 10 μm.

HEK 293 cells expressing the SMAD2-EGFP fusion were treated with various concentration of TGF-beta as indicated, and the redistribution of fluorescence monitored against time. The time profile plots represent increases in fluorescence within the nucleus, normalised to starting values in each cell measured. Each trace is the time profile for a single cell nucleus.

A bar chart representing the end-point change in fluorescence within nuclei (after 850 seconds of treatment) for different concentrations of TGF-beta. Each bar is the value for a single nucleus in each treatment.

Figure 11. The VASP-F64L-S65T-GFP fusion in CHO cells stably transfected with the human insulin receptor. The cells were starved for two hours in HAM F-12 without serum, then treated with 10% foetal calf serum. The image shows the resulting redistribution of fluorescence after 15 minutes of treatment. GFP fluorescence becomes localised in structures identified as focal adhesions along the length of actin stress fibres.

Figure 12. Dose-response relationship for the translocation of PKCα-GFP in BHKhM1 cells stimulated with the muscarininc agonist carbamylcholine using a FLIPR™ to do the actual experiments.

Figure 13. Dose-response relationship for the translocation of PKAc-GFP in CHO/PKAc-F64L-S65T-GFP cells stimulated with forskolin using a FLIPR™ to do the actual experiments.

Figure 14. CHO cells stably expressing the human insulin receptor and mouse cPKA labeled with S65T-GFP were more thoroughly investigated in the FLIPR™ instrument. A forskolin (a substance that increases Adenylate cyclase production of cAMP in the cells) dose-response was created where six separate wells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC (area under the curve) for 9 min of stimulation.

Conclusion: Redistribution of mouse cPKA - BioST can be detected in the FLIPR™ despite the fact that the whole wells, containing around 50-100 000 cells, are illuminated and imaged simultaneously with a spatial resolution that is far from capable of resolving

single cells or subcellular events. The method can be used as a real time measurement of cAMP levels in the cells and as a screening assay to measure effects of ligands to G-protein coupled receptors linked to Gi and Gq type G-proteins.

Figure 15. Dose-response relationship for the disappearance of fluorescence from permeabilised CHO/PKAc-F64L-S65T-GFP when previously exposed to different doses of forskolin.

Figure 16. CHO cells stably expressing the human insulin receptor and human PKC beta 1 labeled with EGFP were investigated in the microscope. A dose-response was created where a set of cells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 4 min of stimulation. From the images the following data were extracted:
Whole image : Just analysing the change in intensity in the whole images taking both cells and background.
Single cell: 5 separate cells were analysed after background compensation. The analysis was made on the entire cell.
Cytoplasm: The same 5 cells as above were analysed after background compensation. the analysis was made on a small region in the cytoplasm close to the nucleus.
Conclusion: Redistribution of human PKC beta 1 – EGFP can only be detected if a subregion of each cell is analysed. The event is clearly visible when the image series is viewed as a movie but if the whole image change in fluorescence or the change in fluorescence in entire cells are analysed the redistribution cannot be detected.

Figure 17. CHO cells stably expressing the human insulin receptor and human PKC beta 1 labeled with EGFP were investigated in the FLIPR™. A dose-response was created where six separate wells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 5 min of stimulation.
Conclusion: Redistribution of human PKC beta 1 – EGFP can be detected in the FLIPR™ despite the fact that the whole wells, containing around 50-100 000 cells, are illuminated and imaged by a detector that has a resolution far below that needed to resolve single cells or subcellular structures. This phenomenon can clearly not be predicted from the microscope data in Figure 16.

Figure 18 CHO cells stably expressing the insulin receptor and a human NFkB – GFP protein hybrid were stimulated with different concentrations of IL-1 for 1 h, then washed with a hypoosmotic buffer (TRIS-base 10mM, MgCl2 2mM, PMSF(Phenyl methyl sulfonyl fluoride) 0.5mM, pH 7.4) and placed on the microscope. A sequence of images were acquired during the addition of 0.05% Triton X-100 and subsequent gentle mixing after a short incubation period. The treatment causes the cell membranes to rupture leaving the fraction of NFkB-GFP that has translocated to the nucleus behind whereas the cytoplasmic amount of the probe leaves the cells more quickly and immediately becomes infinitely diluted in the surrounding medium (out of focus - this part of the total fluorescence from the probe is thereby lost). At a defined time point before and after this treatment a total intensity value for the whole image was extracted. To normalize each experiment, the after value was divided by the before value. meaning that a higher ratio was found in cells where more NFkB had translocated to the nucleus and thereby contributed to the total fluorescence after permeabilisation.

Conclusion: the present protocol is a good example of the possibility of revealing translocation of a fluorescent probe from the cytosol to the nucleus or translocation from the nucleus to the cytosol.

EXAMPLES

EXAMPLE 1 Construction, testing and implementation of an assay for cAMP based on PKA activation.

Useful for monitoring the activity of signalling pathways that lead to altered concentrations of cAMP, e.g. activation of G-protein coupled receptors which couple to G-proteins of the G_S or G_I class.

The catalytic subunit of the murine cAMP dependent protein kinase (PKAc) was fused C-terminally to a F64L-S65T derivative of GFP. The resulting fusion (PKAc-F64L-S65T-GFP) was used for monitoring *in vivo* the translocation and thereby the activation of PKA.

To construct the PKAc-F64L-S65T-GFP fusion, convenient restriction endonuclease sites were introduced into the cDNAs encoding murine PKAc (Gen Bank Accession number: M12303) and F64L-S65T-GFP (sequence disclosed in WO 97/11094) by polymerase chain reaction (PCR). The PCR reactions were performed according to standard protocols with the following primers:

5'PKAc:

TTggACACAAgCTTTggACACCCTCAggATATgggCAACgCCgCCgCCgCCAAg,

3'PKAc:

gTCATCTTCTCgAgTCTTTCAggCgCgCCCAAACTCAgTAAACTCCTTgCCACAC

5'GFP:

TTggACACAAgCTTTggACACggCgCgCCATgAgTAAAggAgAAgAACTTTTC

3'GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT.

The PKAc amplification product was then digested with HindIII+AscI and the F64L-S65T-GFP product with AscI+XhoI. The two digested PCR products were subsequently ligated with a HindIII+XhoI digested plasmid (pZeoSV® mammalian expression vector, Invitrogen, San Diego, CA, USA). The resulting fusion construct (SEQ ID NO:1 and 2) was under control of the SV40 promoter.

Transfection and cell culture conditions:

Chinese hamster ovary cells (CHO), were transfected with the plasmid containing the PKAc-F64L-S65T-GFP fusion using the calcium phosphate precipitate method in HEPES-buffered saline (Sambrook *et al.*, 1989). Stable transfectants were selected using 1000 μg Zeocin/ml (Invitrogen) in the growth medium (DMEM with 1000 mg glucose/l, 10 % fetal bovine serum (FBS), 100 μg penicillin-streptomycin mixture ml^{-1}, 2 mM L-glutamine purchased from Life Technologies Inc., Gaithersburg, MD, USA). Untransfected CHO cells were used as the control. To assess the effect of glucagon on fusion protein translocation, the PKAc-F64L-S65T-GFP fusion was stably expressed in baby hamster kidney cells overexpressing the human glucagon receptor (BHK/GR cells). Untransfected BHK/GR cells were used as the control. Expression of GR was maintained with 500 μg G418/ml (*Neo* marker) andPKAc-F64L-S65T-GFP was maintained with 500 μg Zeocin/ml (*Sh ble* marker). CHO cells were also simultaneously co-transfected with vectors containing the PKAc-F64L-S65T-GFP fusion and the human α2a adrenoceptor (hARa2a).

For fluorescence microscopy, cells were allowed to adhere to Lab-Tek chambered coverglasses (Nalge Nunc Int., Naperville, IL, USA) for at least 24 hours and cultured to about 80% confluence. Prior to experiments, the cells were cultured over night without selection pressure in HAM F-12 medium with glutamax (Life Technologies), 100 μg penicillin-streptomycin mixture ml^{-1} and 0.3 % FBS. This medium has low autofluorescence enabling fluorescence microscopy of cells straight from the incubator.

Monitoring activity of PKA activity in real time:

Image aquisition of live cells were gathered using a Zeiss Axiovert 135M fluorescence microscope fitted with a Fluar 40X, NA: 1.3 oil immersion objective and coupled to a Photometrics CH250 charged coupled device (CCD) camera. The cells were illuminated with a 100 W HBO arc lamp. In the light path was a 470±20 nm excitation filter, a 510 nm dichroic mirror and a 515±15 nm emission filter for minimal image background. The cells were maintained at 37°C with a custom built stage heater.

Images were processed and analysed in the following manner:

Method 1: Stepwise procedure for quantitation of translocation of PKA:

The image was corrected for dark current by performing a pixel-by-pixel subtraction of a dark image (an image taken under the same conditions as the actual image, except the camera shutter is not allowed to open).

The image was corrected for non-uniformity of the illumination by performing a pixel-by-pixel ratio with a flat field correction image (an image taken under the same conditions as the actual image of a uniformly fluorescent specimen).

The image histogram, i.e., the frequency of occurrence of each intensity value in the image, was calculated.

A smoothed, second derivative of the histogram was calculated and the second zero is determined. This zero corresponds to the inflection point of the histogram on the high side of the main peak representing the bulk of the image pixel values.

The value determined in step 4 was subtracted from the image. All negative values were discarded.

The variance (square of the standard deviation) of the remaining pixel values was determined. This value represents the "response" for that image.

Scintillation proximity assay (SPA) for independent quantitation of cAMP.

Method 2: Alternative method for quantitation of PKA redistribution:

The fluorescent aggregates are segmented from each image using an automatically found threshold based on the maximisation of the information measure between the object and background. The *a priori* entropy of the image histogram is used as the information measure.

The area of each image occupied by the aggregates is calculated by counting pixels in the segmented areas.

The value obtained in step 2 for each image in a series, or treatment pair, is normalised to the value found for the first (unstimulated) image collected. A value of zero (0) indicates no redistribution of fluorescence from the starting condition. A value of one (1) by this method equals full redistribution.

Cells were cultured in HAM F-12 medium as described above, but in 96-well plates. The medium was exchanged with Ca^{2+}-HEPES buffer including 100 µM IBMX and the cells were stimulated with different concentrations of forskolin for 10 min. Reactions were stopped with addition of NaOH to 0.14 M and the amount of cAMP produced was measured with the cAMP-SPA kit, RPA538 (Amersham) as described by the manufacturer.

Manipulating intracellular levels of cAMP to test the PKAc-F64L-S65T-GFP fusion.

The following compounds were used to vary cAMP levels: Forskolin, an activator of adenylate cyclase; dbcAMP, a membrane permeable cAMP analog which is not degraded by phosphodiesterase; IBMX, an inhibitor of phosphodiesterase.

CHO cells stably expressing the PKAc-F64L-S65T-GFP, showed a dramatic translocation of the fusion protein from a punctate distribution to an even distribution throughout the cytoplasm following stimulation with 1 μM forskolin (n=3), 10 μM forskolin (n=4) and 50 μM forskolin (n=4) (Fig 1), or dbcAMP at 1mM (n=6).

Fig. 2 shows the progression of response in time following treatment with 1 μM forskolin.

Fig. 3 gives a comparison of the average temporal profiles of fusion protein redistribution and a measure of the extent of each response to the three forskolin concentrations (Fig. 3A, E, B), and to 1 mM dbcAMP (fig 3C) which caused a similar but slower response, and to addition of 100 μM IBMX (n=4, Fig. 3D) which also caused a slow response, even in the absence of adenylate cyclase stimulation. Addition of buffer (n=2) had no effect (data not shown).

As a control for the behaviour of the fusion protein, F64L-S65T-GFP alone was expressed in CHO cells and these were also given 50 μM forskolin (n=5); the uniform diffuse distribution characteristic of GFP in these cells was unaffected by such treatment (data not shown).

The forskolin-induced translocation of PKAc-F64L-S65T-GFP showed a dose-response relationship (Fig 4 and 6), see quantitative procedures above.

Reversibility of PKAc-F64L-S65T-GFP translocation.

The release of the PKAc probe from its cytoplasmic anchoring hotspots was reversible. Washing the cells repeatedly (5-8 times) with buffer after 10μM forskolin treatment completely restored the punctate pattern within 2-5 min (n=2, Fig. 3E). In fact the fusion protein returned to a pattern of fluorescent cytoplasmic aggregates virtually indistinguishable from that observed before forskolin stimulation.

To test whether the return of fusion protein to the cytoplasmic aggregates reflected a decreased $[cAMP]_i$, cells were treated with a combination of 10 μM forskolin and 100 μM IBMX (n=2) then washed repeatedly (5-8 times) with buffer containing 100 μM IBMX (Fig. 3F). In these experiments, the fusion protein did not return to its prestimulatory localisation after removal of forskolin.

Testing the PKA-F64L-S65T-GFP probe with physiologically relevant agents.

To test the probe's response to receptor activation of adenylate cyclase, BHK cells stably transfected with the glucagon receptor and the PKA-F64L-S65T-GFP probe were exposed to glucagon stimulation. The glucagon receptor is coupled to a G_S protein which activates adenylate cyclase, thereby increasing the cAMP level. In these cells, addition of 100 nM glucagon (n=2) caused the release of the PKA-F64L-S65T-GFP probe from the cytoplasmic aggregates and a resulting translocation of the fusion protein to a more even cytoplasmic distribution within 2-3 min (Fig. 3G). Similar but less pronounced effects were seen at lower glucagon concentrations (n=2, data not shown). Addition of buffer (n=2) had no effect over time (data not shown).

Transiently transfected CHO cells expressing hARα2a and the PKA-F64L-S65T-GFP probe were treated with 10 μM forskolin for 7.5 minutes, then, in the continued presence of forskolin, exposed to 10 μM norepinephrine to stimulate the exogenous adrenoreceptors, which couple to a G_i protein, which inhibit adenylate cyclase. This treatment led to reappearance of fluorescence in the cytoplasmic aggregates indicative of a decrease in $[cAMP]_i$ (Fig. 3H).

Fusion protein translocation correlated with $[cAMP]_i$

As described above, the time it took for a response to come to completion was dependent on the forskolin dose (Fig. 5) In addition the degree of responses was also dose-dependent. To test the PKA-F64L-S65T-GFP fusion protein translocation in a semi high through-put system, CHO cells stably transfected with the PKA-F64L-S65T-GFP fusion was stimulated with buffer and 5 increasing doses of forskolin (n=8). Using the image analysis algorithm described above (Method 1), a dose-response relationship was observed in the range from 0.01-50 μM forskolin (Fig. 6). A half-maximal stimulation was observed at about 2 μM forskolin. In parallel, cells were stimulated with buffer and 8 increasing concentrations of forskolin (n=4) in the range 0.01-50 μM. The amount of cAMP produced was measured in an SPA assay. A steep increase was observed between 1 and 5 μM forskolin coincident with the steepest part of the curve for fusion protein translocation (also Fig. 6).

EXAMPLE 2 Probe for detection of PKC activity

Construction of PKC-GFP fusion:

The probe was constructed by ligating two restriction enzyme treated polymerase chain reaction (PCR) amplification products of the cDNA for murine PKCα (GenBank

Accession number: M25811) and F64L-S65T-GFP (sequence disclosed in WO 97/11094) respectively. Taq® polymerase and the following oligonucleotide primers were used for PCR;

5'mPKCα:

TTggACACAAgCTTTggACACCCTCAggATATggCTgACgTTTACCCggCCAACg

3'mPKCα:

gTCATCTTCTCgAgTCTTTCAggCgCgCCCTACTgCACTTTgCAAgATTgggTgC,

5'F64L-S65T-GFP:

TTggACACAAgCTTTggACACggCgCgCCATgAgTAAAggAgAAgAACTTTTC,

3'F64L-S65T-GFP:

gTCATCTTCTCgAgTCTTACTCCTgAggTTTgTATAgTTCATCCATgCCATgT.

The hybrid DNA strand was inserted into the pZeoSV® mammalian expression vector as a HindIII-XhoI casette as described in example 1.

BHK cells expressing the human M1 receptor under the control of the inducible metallothionine promoter and maintained with the dihydrofolate reductase marker were transfected with the PKCα-F64L-S65T-GFP probe using the calcium phosphate precipitate method in HEPES buffered saline (HBS [pH 7.10]). Stable transfectants were selected using 1000 µg Zeocin®/ml in the growth medium (DMEM with 1000 mg glucose/l, 10 % foetal bovine serum (FBS), 100 µg penicillin-streptomycin mixture ml-1, 2 mM l-glutamine). The hM1 receptor and PKCα-F64L-S65T-GFP fusion protein were maintained with 500 nM methotrexate and 500 µg Zeocin®/ml respectively. 24 hours prior to any experiment, the cells were transferred to HAM F-12 medium with glutamax, 100 µg penicillin-streptomycin mixture ml^{-1} and 0.3 % FBS. This medium relieves selection pressure, gives a low induction of signal transduction pathways and has a low autofluorescence at the relevant wavelength enabling fluorescence microscopy of cells straight from the incubator.

Method 1: Monitoring the PKCα activity in real time:

Digital images of live cells were gathered using a Zeiss Axiovert 135M fluorescence microscope fitted with a 40X, NA: 1.3 oil immersion objective and coupled to a Photometrics CH250 charged coupled device (CCD) camera. The cells were illuminated with a 100 W arc lamp. In the light path was a 470±20 nm excitation filter, a 510 nm dichroic mirror and a 515±15 nm emission filter for minimal image background. The cells were kept and monitored to be at 37°C with a custom built stage heater.

Images were analyzed using the IPLab software package for Macintosh.

Upon stimulation of the M1-BHK cells, stably expressing the PKCα-F64L-S65T-GFP fusion, with carbachol we observed a dose-dependent transient translocation from the cytoplasm to the plasma membrane (Fig. 7a,b,c). Simultaneous measurement of the cytosolic free calcium concentration shows that the carbachol-induced calcium mobilisation precedes the translocation (Fig. 8).

Stepwise procedure for quantification of translocation of PKCα:

The image was corrected for dark current by performing a pixel-by-pixel subtraction of a dark image (an image taken under the same conditions as the actual image, except the camera shutter is not allowed to open).

The image was corrected for non-uniformity of the illumination by performing a pixel-by-pixel ratio with a flat field correction image (an image taken under the same conditions as the actual image of a uniformly fluorescent specimen).

A copy of the image was made in which the edges are identified. The edges in the image are found by a standard edge-detection procedure – convolving the image with a kernel which removes any large-scale unchanging components (i.e., background) and accentuates any small-scale changes (i.e., sharp edges). This image was then converted to a binary image by threshholding. Objects in the binary image which are too small to represent the edges of cells were discarded. A dilation of the binary image was performed to close any gaps in the image edges. Any edge objects in the image which were in contact with the borders of the image are discarded. This binary image represents the edge mask.

Another copy of image was made via the procedure in step 3. This copy was further processed to detect objects which enclose "holes" and setting all pixels inside the holes to the binary value of the edge, i.e., one. This image represents the whole cell mask.

The original image was masked with the edge mask from step 3 and the sum total of all pixel values is determined.

The original image was masked with the whole cell mask from step 4 and the sum total of all pixel values was determined.

The value from step 5 was divided by the value from step 6 to give the final result, the fraction of fluorescence intensity in the cells which was localized in the edges.

EXAMPLE 3 Probes for detection of mitogen activated protein kinase Erk1 redistribution.

Useful for monitoring signalling pathways involving MAPK, e.g. to identify compounds which modulate the activity of the pathway in living cells.

Erk1, a serine/threonine protein kinase, is a component of a signalling pathway that is activated by e.g. many growth factors.

Probes for detection of ERK-1 activity in real time within living cells:

The extracellular signal regulated kinase (ERK-1, a mitogen activated protein kinase, MAPK) is fused N- or C-terminally to a derivative of GFP. The resulting fusions expressed in different mammalian cells are used for monitoring *in vivo* the nuclear translocation, and thereby the activation, of ERK1 in response to stimuli that activate the MAPK pathway.

The human Erk1 gene (GenBank Accession number: X60188) was amplified using PCR according to standard protocols with primers

Erk1-top

5'-TAGAATTCAACCATGGCGGCGGCGGCGGCG-3'

and Erk1-bottom/+stop

5'-TAGGATCCCTAGGGGGCCTCCAGCACTCC-3'.

The PCR product was digested with restriction enzymes EcoR1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with EcoR1 and BamH1. This produces an EGFP-Erk1 fusion (SEQ ID NOs: 5 and 6) under the control of a CMV promoter.

The plamid containing the EGFP-Erk1 fusion was transfected into HEK293 cells employing the FUGENE transfection reagent (Boehringer Mannheim). Prior to experiments the cells were grown to 80%-90% confluency 8 well chambers in DMEM with 10% FCS. The cells were washed in plain HAM F-12 medium (without FCS), and then incubated for 30-60 minutes in plain HAM F-12 (without FCS) with 100 micromolar PD98059, an inhibitor of MEK1, a kinase which activates Erk1; this step effectively empties the nucleus of EGFP-Erk1. Just before starting the experiment, the HAM F-12 was replaced with Hepes buffer following a wash with Hepes buffer. This removes the PD98059 inhibitor; if blocking of MEK1 is still wanted (e.g. in control experiments), the inhibitor is included in the Hepes buffer.

The experimental setup of the microscope was as described in example 1.

60 images were collected with 10 seconds between each, and with the test compound added after image number 10.

Addition of EGF (1-100 nM) caused within minutes a redistribution of EGFP-Erk1 from the cytoplasm into the nucleus (Fig. 9a,b).

The response was quantitated as described below and a dose-dependent relationship between EGF concentration and nuclear translocation of EGFP-Erk1 was found (Fig. 9c,d). Redistribution of GFP fluorescence is expressed in this example as the change in the ratio value between areas in nuclear versus cytoplasmic compartments of the cell. Each time profile is the average of nuclear to cytoplasmic ratios from six cells in each treatment.

EXAMPLE 4 Probes for detection of Smad2 redistribution.

Useful for monitoring signalling pathways activated by some members of the transforming growth factor-beta family, e.g. to identify compounds which modulate the activity of the pathway in living cells.

Smad 2, a signal transducer, is a component of a signalling pathway that is induced by some members of the TGFbeta family of cytokines.

a) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers

Smad2-top

5'-GTGAATTCGACCATGTCGTCCATCTTGCCATTC-3'

and Smad2-bottom/+stop

5'-GTGGTACCTTATGACATGCTTGAGCAACGCAC-3'.

The PCR product was digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-C1 (Clontech; Palo Alto; GenBank Accession number U55763) digested with EcoR1 and Acc65I. This produces an EGFP-Smad2 fusion (SEQ ID NOs: 7 and 8) under the control of a CMV promoter.

b) The human Smad2 gene (GenBank Accession number: AF027964) was amplified using PCR according to standard protocols with primers

Smad2-top

5'-GTGAATTCGACCATGTCGTCCATCTTGCCATTC-3'

and Smad2-bottom/-stop

5'-GTGGTACCCATGACATGCTTGAGCAACGCAC-3'.

The PCR product was digested with restriction enzymes EcoR1 and Acc65I, and ligated into pEGFP-N1 (Clontech, Palo Alto; GenBank Accession number U55762) digested with EcoR1 and Acc65I. This produces a Smad2-EGFP fusion (SEQ ID NOs:9 and 10) under the control of a CMV promoter.

The plasmid containing the EGFP-Smad2 fusion was transfected into HEK293 cells, where it showed a cytoplasmic distribution. Prior to experiments the cells were grown in 8 well Nunc chambers in DMEM with 10% FCS to 80% confluence and starved overnight in HAM F-12 medium without FCS.

For experiments, the HAM F-12 medium was replaced with Hepes buffer pH 7.2. The experimental setup of the microscope was as described in example 1. 90 images were collected with 10 seconds between each, and with the test compound added after image number 5.

After serum starvation of cells, each nucleus contains less GFP fluorescence than the surrounding cytoplasm (Fig. 10a). Addition of TGFbeta caused within minutes a redistribution of EGFP-Smad2 from the cytoplasma into the nucleus (Fig. 10b).

The redistribution of fluorescence within the treated cells was quantified simply as the fractional increase in nuclear fluorescence normalised to the starting value of GFP fluorescence in the nucleus of each unstimulated cell and displayed a dose dependent change in response to TGFβ (fig. 10c).

EXAMPLE 5 Probes for detection of VASP redistribution.

Useful for monitoring signalling pathways involving rearrangement of cytoskeletal elements, e.g. to identify compounds which modulate the activity of the pathway in living cells. VASP, a phosphoprotein, is a component of cytoskeletal structures, which redistributes in response to signals that affect focal adhesions.

The human VASP gene (GenBank Accession number: Z46389) was amplified using PCR according to standard protocols with primers

VASP-top

5'-GGGAAGCTTCCATGAGCGAGACGGTCATC-3'

and VASP-bottom/+stop

5'-CCCGGATCCTCAGGGAGAACCCCGCTTC-3'.

The PCR product was digested with restriction enzymes Hind3 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Hind3and BamH1. This produces an EGFP-VASP fusion (SEQ ID NOs:11 and 12) under the control of a CMV promoter.

The resulting plasmid was transfected into CHO cells expressing the human insulin receptor using the calcium-phosphate transfection method. Prior to experiments, cells were grown in 8 well Nunc chambers and starved overnight in medium without FCS. Experiments are performed in a microscope setup as described in example 1.10% FCS was added to the cells and images were collected. The EGFP-VASP fusion was

redistributed from a somewhat even distribution near the periphery into more localised structures, identified as focal adhesion points (Fig. 11).

EXAMPLE 7 Probes for detection of NFkappaB redistribution.

Useful for monitoring signalling pathways leading to activation of NFkappaB, e.g. to identify compounds which modulate the activity of the pathway in living cells. NFkappaB, an activator of transcription, is a component of signalling pathways that are responsive to a varity of inducers including cytokines, lymphokines, and some immunosuppressive agents.

a) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers
NFkappaB-top
5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC-3'
and NFkappaB-bottom/+stop
5'-GTGGATCCTTAGGAGCTGATCTGACTCAGCAG-3'.
The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Xho1 and BamH1. This produces an EGFP-NFkappaB fusion (SEQ ID NOs:13 and 14) under the control of a CMV promoter.

b) The human NFkappaB p65 subunit gene (GenBank Accession number: M62399) is amplified using PCR according to standard protocols with primers
NFkappaB-top
5'-GTCTCGAGCCATGGACGAACTGTTCCCCCTCATC-3'
and NFkappaB-bottom/-stop
5'-GTGGATCCAAGGAGCTGATCTGACTCAGCAG-3'.
The PCR product is digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-N1 (Clontech, Palo Alto; GenBank Accession number U55762) digested with Xho1 and BamH1. This produces an NFkappaB-EGFP fusion (SEQ ID NOs: 15 and 16) under the control of a CMV promoter.

The resulting plasmids are transfected into a suitable cell line, e.g. Jurkat, in which the EGFP-NFkappaB probe and/or the NFkappaB-EGFP probe should change its cellular distribution from cytoplasmic to nuclear in response to activation of the signalling pathway with e.g. IL-1.

CHO cells stably expressing the insulin receptor and a human NFkB – GFP protein hybrid were stimulated with different concentrations of IL-1 for 1 hour, then washed with a hypoosmotic buffer (TRIS-base 10mM, MgCl2 2mM, PMSF(Phenyl methyl sulfonyl fluoride) 0.5mM, pH 7.4) and placed on the microscope. A sequence of images were acquired during the addition of 0.05% Triton X-100 and subsequent gentle mixing after a short incubation period. The treatment causes the cell membranes to rupture leaving the fraction of NFkB-GFP that has translocated to the nucleus behind whereas the cytoplasmic amount of the probe leaves the cells more quickly and immediately becomes infinitely diluted in the surrounding medium (out of focus - this part of the total fluorescence from the probe is thereby lost). At a defined time point before and after this treatment a total intensity value for the whole image was extracted. To normalize each experiment, the after value was divided by the before value, meaning that a higher ratio was found in cells where more NFkB had translocated to the nucleus and thereby contributed to the total fluorescence after permeabilisation. the actual data from such an experiment run in duplicate is shown in Figure 18.

Conclusion: the present protocol is a good example of the possibility of revealing translocation of a fluorescent probe from the cytosol to the nucleus or translocation from the nucleus to the cytosol by using a measurement immediately before and after plasma membrane permeabilisation recorded a s an image sequence.

EXAMPLE 8 real-time redistribution of protein kinase C α

Measurement of the real-time redistribution of protein kinase C α isoform-GFP fusion (PKCα-GFP, SEQ ID NOs: 3 and 4) in response to carbamylcholine stimulation of the muscarinic M1 receptor; 96 parallel redistribution measurements in microtiter plates.

BHK cells were stably expressing a recombinant human muscarinic type 1 receptor, under the selection with 500 μg/ml Methotrexate, and also a PKCα-GFP construct (KaA 048), under the selection of 500 nM Zeocin. The cells were grown in 96-well plates (Packard ViewPlate, black with transparent bottom), washed and preincubated in a Hank's Buffered Salt solution (HBSS) without phenol red, with 20 mM HEPES and 5.5 mM glucose.

The plate was measured in a FLIPR™ (Fluorescence Imaging Plate Reader) from Molecular Devices. The 488 nm emission line from an argon ion laser, run at between 0.4 and 0.8 W output, was used to excite fluorescence form the GFP. Emission wavelengths were collected through a 510 to 565 nm band pass filter.

The cells were challenged with three doses of carbamylcholine, an M1 receptor agonist known from previous studies to give a microscopically detectable redistribution of the PKCα-GFP construct [(Almholt *et al.* 1997)]. Measurements were made every 10 seconds for 5 minutes. After data handling including normalisation of baseline fluorescence for the different wells. background subtraction and averaging the 6 wells used for each concentration the data presented in figure 14 were obtained. It can clearly be seen (Fig 12) that carbamylcholine gave a time- and dose-dependent, and transient, decrease in fluorescence very similar to the time- and dose-dependent profile seen in microscopic fluorescence measurements [(see Almholt *et al.* 1997)]. This experiment was repeated twice on the same batch of cells with similar results.

EXAMPLE 9 real-time redistribution of cyclic-AMP dependent protein kinase catalytic subuit-GFP fusion

Measurement of the real-time redistribution of cyclic-AMP dependent protein kinase catalytic subuit-GFP fusion (C-GFP^{LT} SEQ ID NOs: 1 and 2) in response to forskolin stimulation of the adenylate cyclase; 96 parallel redistribution measurements in microtiter plates.

CHO cells were stably transfected with hybrid DNA for the PKA catalytic subunit-F64L+S65T GFP (C-GFP^{LT}) fusion protein, and were typically under continuous selection with 1000 µg/ml zeocin (Invitrogen). The cells were grown without selection for 2 days in 96-well plates (Packard ViewPlate, black with transparent bottom), washed and preincubated in a Hank's Buffered Salt solution (HBSS) without phenol red, with 20 mM HEPES and 5.5 mM glucose.

The plate was measured in a FLIPR™ (Fluorescence Imaging Plate Reader) from Molecular Devices. The 488 nm emission line from an argon ion laser, run at between 0.4 and 0.8 W output, was used to excite fluorescence from the GFP. Emission wavelengths were collected through a 510 to 565 nm band pass filter.

The cells were challenged with three doses of forskolin (Fig 13), an adenylate cyclase agonist known from previous studies to give a microscopically detectable redistribution of the C-GFP^{LT} construct. Measurements were made every 10 seconds for over 6 minutes from the point of addition of forskolin. After data handling including normalisation of baseline fluorescence for the different wells, background subtraction and averaging the 6 wells used for each concentration the data presented below were obtained. It can clearly be seen in figure 15 that forskolin gave a time- and dose-dependent decrease in fluorescence very similar to the time- and dose-dependent profile seen in microscopic

fluorescence measurements. This experiment was repeated twice on the same batch of cells with similar results. As can be seen in figure 14, a more extensive dose-response test gives at hand that this method is both sensitive and reproducible enough to use as the basis for a high throughput screening assay.

EXAMPLE 10 cyclic-AMP dependent protein kinase catalytic subuit-GFP fusion

Measurement of the redistribution response of cyclic-AMP dependent protein kinase catalytic subuit-GFP fusion (C-GFP^{LT} SEQ ID NOs: 1 and 2) after forskolin stimulation of the adenylate cyclase; measurement of the change in total fluorescence upon permeabilisation of agonist-treated cells.

CHO cells were stably transfected with hybrid DNA for the PKA catalytic subunit-F64L+S65T GFP (C-GFP^{LT}) fusion protein, and were typically under continuous selection with 1000 μg/ml zeocin (Invitrogen). For the experiments reported here, cells were grown without selection to 90% confluence in 8-well tissue culture-treated Lab-Tek® chambered coverglass units (chambers, obtained from Nunc, Inc. Illinois, USA). Immediately prior to the experiment growth medium was washed from the cells and replaced with 200 μl HEPES buffer per well.

For the results reported here, chambers were measured using a cooled CCD camera (KAF1400 chip, Photometrics Ltd., USA) attached to an inverted microscope (Diaphot 300, Nikon, Japan) equipped with a x40 oil-immersion Fluar lens, NA 1.4. Cells were illuminated with 450-490 nm light from a 50 W HBO lamp, and emitted light collected between 510-560 nm.

The cells were challenged with four doses of forskolin, an adenylate cyclase agonist known from previous studies to give a microscopically detectable redistribution of the C-GFP^{LT} construct. Images were collected at 10-second intervals for a period of 10 minutes for each treatment. Six minutes after the addition of forskolin or buffer control, Triton-X100 was added to a final concentration of 0.1%. The detergent releases freely mobile C-GFP^{LT} from the cells. The change in fluorescence resulting from this loss was measured after 1 minute of equilibration. After data handling including background subtraction and normalisation to pre-detergent values, the data presented in figure 16 were obtained. Permeabilisation caused decreases in fluorescence, the magnitude of which were dependent on the forskolin treatments. This experiment was repeated twice on the same batch of cells with similar results.

EXAMPLE 11 Probes for detection of PKCβ1 redistribution.

Useful for monitoring signalling pathways involving Protein Kinase C, e.g. to identify compounds which modulate the activity of the pathway in living cells.

PKCbeta1, a serine/threonine protein kinase, is closely related to PKCalpha and PKCbeta2 but not identical; it is a component of a signalling pathway which is activated by elevation of intracellular calcium concomitant with an increase in diacylglycerol species.

SUB B7

a) The human PKCbeta1 gene (GenBank Accession number: X06318) was amplified using PCR according to standard protocols with primers

PKCβ1-top

GTCTCGAGGCAAGATGGCTGACCC

and PKCβ1-bottom

GTGGATCCCTACACATTAATGACAAACTCTGGG.

The PCR product was digested with restriction enzymes Xho1 and BamH1, and ligated into pEGFP-C1 (Clontech, Palo Alto; GenBank Accession number U55763) digested with Xho1 and BamH1. This produces an EGFP-PKCβ1 fusion (SEQ ID NOs: 17 and 18) under the control of a CMV promoter.

b) CHO cells stably expressing the human insulin receptor and human PKC beta 1 labeled with EGFP were investigated in the microscope. A dose-response was created where a set of cells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 4 min of stimulation.

It can be seen in figure 16 that using microscopic measurements, redistribution of human PKC beta 1 – EGFP can only be detected if a subregion of each cell is analysed. The event is clearly visible when the image series is viewed as a movie but if the whole image changes in fluorescence or the changes in fluorescence in entire cells are analysed the redistribution cannot be detected.

CHO cells stably expressing the human insulin receptor and human PKC beta 1 labelled with EGFP were investigated in the FLIPR™. A dose-response was created where six separate wells were imaged over time for each concentration. The changes in fluorescence were calculated as AUC for 5 min of stimulation. As shown in figure 17 redistribution of human PKC beta 1 – EGFP can be detected in the FLIPR™ instrument despite the fact that the whole wells, containing around 50-100 000 cells, are illuminated and imaged with a resolution far below what is needed to resolve single cells or subcellular compartments. This phenomenon can clearly not be predicted from the

microscope data in Figure 16. Based on these observations it is clear that a screening assay can be established in the FLIPR™ instrument. It might even be possible to establish a high throughput screening assay with further optimisation.

REFERENCES:

Adams, S.R., Harootunian, A.T., Buechler, Y.J., Taylor, S.S. & Tsien, R.Y. (1991) Nature **349**, 694-697.

Barak, L.S., Ferguson, S.S.G., Zhang, J. & Caron, M.G. (1997) J. Biol. Chem. **272**:44, 27497-27500.

Bastiaens, P.I.H. & Jovin, T.M. (1996) Proc. Natl. Acad. Sci. USA **93**, 8407-8412.

Beals, C.R., Clipstone, N.A., Ho, S.N. & Crabtree, G.R. (1998) Genes and Development **11**:7, 824-834.

Blobe, G.C., Stribling, D.S., Fabbro, D., Stabel, S & Hannun, Y.A. (1996) J. Biol. Chem. **271**, 15823-15830.

Carey, K.L., Richards, S.A., Lounsbury, K.M. & Macara, I.G. (1996) J. Cell Biol. **133**: 5, 985-996.

Chalfie, M., Tu, Y., Euskirchen, G.. Ward, W.W. & Prasher, D.C. (1994) Science **263**, 802-805.

Cossette, L.J., Hoglinger, O., Mou, L.J. & Shen, S.H. (1997) Exp. Cell Res. **223**, 459-466.

DeBernardi, M.A. & Brooker, G. (1996) Proc. Natl. Acad. Sci. USA **93**, 4577-4582.

Farese, R.V. (1992) Biochem. J. **288**, 319-323.

Fulop Jr., T., Leblanc, C., Lacombe, G. & Dupuis, G. (1995) FEBS Lett. **375**, 69-74.

Georget, V., Lobaccaro, J.M., Terouanne, B., Mangeat, P., Nicolas, J.C. & Sultan, C. (1997) Mol. Cell. Endocrinol. **129**:1, 17-26.

Godson, C., Masliah, E., Balboa, M.A., Ellisman, M.H. & Insel, P.A. (1996) Biochem. Biophys. Acta **1313**, 63-71.

Guiliano, K.A., DeBiasio, R, Dunlay, R.T., Gough, A., Volosky, J.M., Zock, J., Pavlakis, G.N. & Taylor, D.L. (1997) J. Biomol. Screening **2**:4, 249-259.

Khalil, R.A., Lajoie, C., Resnick, M.S. & Morgan, K.G. (1992) Am. J. Physiol. **263** (Cell Physiol. 32) C714-C719.

Oancea, E., Teruel, M.N., Quest, A.F.G. & Meyer, T. (1998) J. Cell Biol. **140**:3, 485-498.

Sano, M., Kohno, M. & Iwanaga, M. (1995) Brain Res. **688**, 213-218.

Sakai, N., Sasaki, K., Hasegawa, C., Ohkura, M., Sumioka, K., Shirai, Y. & Saito, N. (1996) Soc. Neuroscience 22, 69P (Abstract).

Sakai, N., Sakai, K. Hasegawa, C.. Ohkura, M., Sumioka, K., Shirai, Y., & Naoaki, S. (1997) Japanese Journal of Pharmacology 73, 69P (Abstract of a meeting held 22-23 March).

Schmidt, D.J., Ikebe, M., Kitamura, K. & Fay, F.S. (1997) FASEB J. 11, 2924 (Abstract).

Schroeder, K. & Neagle, B.J. (1996) Biomolecular Screening 1, 75-80.

Silverman, L., Campbell, R. & Broach, J.R. (1998) Current Opinion in Chemical Biology 2:3, 397-403.

Stauffer, T.P., Ahn, S. & Meyer, T. (1998) Current Biol. 8:6, 343-346.